Influence of Feed Supplements on the Porcine Intestinal *Escherichia coli* Microbiota

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the Department of Biology, Chemistry and Pharmacy
of the Freie Universität Berlin

by

Carmen Maria Bednorz
from Berlin

February 2015
Berlin

The work for this thesis was performed between May 2010 and June 2014 at the Insitute of Microbiology and Epizootics at the Department of Veterinary Medicine at the Freie Universität Berlin. My thesis was supervised by Prof. Dr. Lothar H. Wieler and Prof. Dr. Rupert Mutzel.

1st Reviewer: Prof. Dr. Lothar H. Wieler
Freie Universität Berlin
Fachbereich Veterinärmedizin
Institut für Mikrobiologie und Tierseuchen
Robert-von-Ostertag-Str. 7-13
14163 Berlin

2nd Reviewer: Prof. Dr. Rupert Mutzel
Freie Universität Berlin
Fachbereich Biologie, Chemie und Pharmazie
Institut für Biologie
Königin-Luise-Str. 12-16
14195 Berlin

Date of defense: May 18th 2015

In Liebe und Dankbarkeit für meine Eltern

Danksagung

Meinem Doktorvater **Prof. Dr. Lothar H. Wieler** möchte ich für die Möglichkeit danken, dass ich am Institut für Mikrobiologie und Tierseuchen promovieren und an diesem interessanten Thema arbeiten durfte. Ich danke Ihnen, dass Sie stets ein offenes Ohr hatten und mich in allen fachlichen Fragen und Problemstellungen unterstützt haben. Die vielen Gespräche über das Projekt haben mir sehr geholfen es in dieser Form abzuschließen.

Prof. Dr. Rupert Mutzel danke ich als Zweitgutachter für die ständige Ansprechbarkeit und die immer offene Bürotür.

Dr. Sebastian Günther danke ich für die stetige Unterstützung bei anwendungstechnischen Fragen im Labor und Problemlösungen. Zudem möchte ich mich für die Ratschläge beim Schreiben der Publikationen sowie das kritische Lesen dieser Arbeit bedanken. **Dr. Astrid Bethe** danke ich für ihre Unterstützung bei Fragen und Unklarheiten. Ich konnte sie jederzeit ansprechen und sie hatte bei Problemen immer ein offenes Ohr für mich.

Der Deutschen Forschungsgemeinschaft danke ich für die finanzielle Unterstützung, sowie dem **Sonderforschungsbereich 852** und der **Dahlem Research School** (DRS) für die Möglichkeit zur Weiterbildung an der Research School und der Teilnahme am PhD Programm. **Dr. Gunnar Loh** danke ich für das Mentoring innerhalb des PhD Programms der DRS.

Kathrin Oelgeschläger und **Bianca Kinnemann** danke ich für ihre tolle Zusammenarbeit, die Hilfe im Labor und ihre hohe Einsatzbereitschaft für das Projekt. **Monica Feldhan** danke ich für Unterstützung bei der Herstellung von den zahlreichen Nährböden und der ständigen Hilfestellung bei Fragen dazu. Ich möchte auch **Petra Krinke** und **Andrea Schmidt** für ihre Hilfe im Labor danken.

Meine lieben Doktorandenkollegen **Daniela, Feri, Inga, Katharina, Lisa, Nina, Sarah, Stefan, Steffi, Susi, Szilvi, Yassmin und Yun** danke ich für eine schöne und lustige Zeit während der Kaffeerunden und einem offenen Ohr, wenn es Probleme jeglicher Art gab. Meinen Freunden Jessi und Basti danke ich für die seelische Unterstützung in dieser Zeit.

Meiner geliebten Familie, insbesondere **meinen Eltern und meiner Schwester** danke ich für die ständige Unterstützung und den Rückhalt, den sie mir gegeben haben. Ohne euch und eure Unterstützung während des Studiums und besonders während der Promotionszeit,

wäre das erfolgreiche Abschließen der Dissertation in dieser Form niemals möglich gewesen. In ausweglosen Situationen habt ihr mich durch euren Zuspruch aufgebaut und mir die nötige Kraft gegeben um nach vorne zu schauen, das Ziel weiterhin vor Augen zu haben und alle Widrigkeiten auszublenden. Ohne Euch hätte ich es nicht geschafft diese Arbeit fertigzustellen. DANKE!

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List of Abbreviations

ADD	Agar Disc Diffusion test
AEEC	attaching and effacing *Escherichia coli*
aEPEC	atypical enteropathogenic *Escherichia coli*
AIDA	“adhesin involved in diffuse adherence”
Amp	ampicilin
APEC	avian pathogenic *Escherichia coli*
CDF	cation diffusion facilitator
Cefo	cefotaxim
cfu	colony forming units
CRC	Collaborative Research Center
d	day(s)
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EcN	*Escherichia coli* Nissle
e.g.	for example
EHEC	Enterohaemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	extraintestinal pathogenic *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
g	gram
GI tract	gastrointestinal tract

h	hour(s)
HGT	Horizontal Gene Transfer
ICE	integrative conjugative elements
i.e.	that is
IgA	immunoglobulin A
IgM	immunoglobulin M
IL-8	Interleukin
InPEC	intestinal pathogenic *Escherichia coil*
kb	kilo base pairs
l	liter
LAB	Lactic Acid Bacteria
LEE	locus of enterocyte effacement
Mb	mega base pairs
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
mol	molar
mRNA	messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
n	number of tested individuals
NMEC	newborn meningitis-causing *Escherichia coli*
PAI	Pathogenicity Island
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field-Gel-Electrophoresis

ppm	parts per million
PWD	post-weaning diarrhea
RNA	ribonucleic acid
SePEC	septicemia-associated *Escherichia coli*
SIV	swine influenza virus
SNP	single nucleotide polymorphism
spp.	species
ST	sequence type
STEC	Shiga-toxin producing *Escherichia coli*
Strep	streptomycin
Tetra	tetracycline
TGEV	transmissible gastroenteritis-virus
™	trademark
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
VAGs	virulence-associated genes
WHO	World Health Organization
ZnO	zinc oxide
®	registered
~	approximately

1. Introduction

1.1. General Background

Feed supplements have become highly important in livestock breeding, especially since the European Union prohibited antibiotic growth promoters in 2006 *(EC, 2005).* As a result of this, probiotics and divalent metal cations are commonly used as supplements in animal farming. They are known to promote the growth and health of the supplemented animals *(Carlson et al., 2008; Fuller, 1989; Lilly and Stillwell, 1965).*

The Collaborative Research Centre 852 (CRC 852) "NUTRITION AND INTESTINAL MICROBIOTA - HOST INTERACTION IN THE PIG" focused on this topic aiming to elucidate the effects and mechanisms of feeding probiotics and zinc as metal ion on the microbiota and the immune response of pigs. The CRC 852 concentrated on the supplementation of pigs especially due to the fact that pigs represent a large proportion of livestock breeding in Germany.

Under the umbrella of the CRC 852 two major animal feeding experiments were performed using young piglets:

i. *Enterococcus (E.) faecium* NCIMB 10415 supplementation before and after weaning over a period of six weeks (12 – 56 days) with 5.1 x 10^6 CFU/g (prestarter) and 3.6 x 10^6 CFU/g (starter) of *E. faecium* NCIMB 10415
ii. Zinc supplementation with different concentrations after weaning over a period of four weeks with three feeding groups (50 ppm (control), 150 ppm (low zinc) and 2,500 ppm (high zinc))

This thesis is a part of the CRC 852 (Subproject A3) and focuses on the influence of the above mentioned feed supplements on the enteric *Escherichia (E.) coli* population in pigs.
E. coli is a member of the gastrointestinal autochthonous microbiota of birds and mammals including pigs, and contributes to the maintenance of the microbial gut balance *(Gordon and Cowling, 2003).* However, *E. coli* is also one of the most important intestinal pathogens in pig production, causing high economic losses *(Wieler et al., 2001).* It is a Gram-negative, facultative anaerobic bacterium with a highly dynamic and individual population structure *(Katouli et al., 1995; Katouli et al., 1999; Schierack et al., 2009b; Schierack et al., 2006).* As mentioned above although a member of the commensal

microbiota, *E. coli* can also be pathogenic and is therefore classified into various pathotypes. These *E. coli* pathogens often cause diarrhea or other diseases *(Ewers et al., 2007; Fairbrother et al., 2005; Kaper et al., 2004; Nagy and Fekete, 2005).*

The upcoming chapter will give an overview of the role of *E. coli* in the microbiota of pigs, both as commensal and pathogen. This is necessary in order to provide an understanding of the importance of the presented study. Current knowledge in the literature about the feed supplements *E. faecium* and zinc will provide a first overview of the known effects on the *E. coli* population in pigs. The concept of this thesis will be explained through the aims of this project and the overall design of the study.

1.2. *E. coli* in pigs

E. coli is a well characterized microorganism and a part of the gastrointestinal autochthonous microbiota of many mammals and birds *(Gordon et al., 2003).* The following information concentrates on the knowledge required for a better understanding of the important role of *E. coli* in the microbiota, especially in that of pigs.

1.2.1. The bacterium *E. coli*

E. coli belongs to the family Enterobacteriaceae and is one of most important representatives of this family which consists of several genera and species that colonize the small and large intestine. *E. coli* is a Gram-negative, non spore-forming, rod-shaped, facultative anaerobic microorganism. It is estimated that 1 to 4% of all cultivable bacteria of the colon are *E. coli* and that up to 10^{10} *E. coli* can be detected in one gram of feces *(Scharek et al., 2005; Selander et al., 1987).* In general, *E. coli* can be classified as commensal or as one of various pathogenic types. Pathogenic *E. coli* are divided into intestinal pathogenic *E. coli* (InPEC), which cause intestinal diseases like diarrhea (EHEC, EPEC, ETEC and EAEC), and extraintestinal pathogenic *E. coli* (ExPEC), which cause extraintestinal diseases, such as an urinary tract infection or mastitis *(Ewers et al., 2009; Ewers et al., 2007; Fairbrother et al., 2005; Kaper et al., 2004; Nagy et al., 2005).*

The genome of *E. coli* is very heterogeneous, with a genome size varying from 4.5 to 5.5 Mb *(Bergthorsson and Ochman, 1998; Ochman and Jones, 2000; Snipen and Ussery, 2012).* This is due to the fact that it consists of a conserved core genome of about 2,000 genes and an accessory genome of roughly 18,000 genes *(Lukjancenko et al., 2010; Rasko*

et al., 2008; Touchon et al., 2009). The core genome represents the conserved part and harbors the genes for elementary cell functions. The accessory genome is the variable part of the bacterial genome and is responsible for high levels of genetic flux *(Medini et al., 2005; Touchon et al., 2009).* The accessory genes thus make up more than 90% of the accessory genome and about 80% of a typical genome *(Lukjancenko et al., 2010).* Particularly virulence, antibiotic resistance genes, or genes with other selection advantages are often located in this region *(Dobrindt et al., 2002; Kaper et al., 2004; Rasko et al., 2008). E. coli* has an open accessory genome that is still evolving through gene acquisition and diversification *(Rasko et al., 2008).* Such changes in the genomic content are often caused by the exchange of mobile genetic elements *(Nataro and Kaper, 1998).* In *E. coli* insertion and deletion takes place between a few base pairs of up to a size of 100 kb *(Hacker and Kaper, 2000; Perna et al., 2001; Rode et al., 1999).* Mobile DNA elements are: "genomic islands," bacteriophages, plasmids, integrative conjugative elements (ICE), IS elements, transposons, and integrons *(Dobrindt and Hacker, 2001; Wozniak and Waldor, 2010).* They can be located either as part of the chromosome or as extra-chromosomal elements *(Dobrindt, 2005; Dobrindt et al., 2002; Dobrindt et al., 2004; Wozniak et al., 2010).*

Different pathogenic *E. coli* strains harbor individual virulence-associated factors. These factors are often lacking in non-pathogenic strains. Several mechanisms for acquiring virulence-associated genes (VAGs) via horizontal gene transfer (HGT) exist, like transformation, transduction, and conjugation.

HGT is important for genome plasticity *(Treangen and Rocha, 2011).* The most effective mechanism of HGT is conjugation, the exchange of plasmids *(Hehemann et al., 2010; Nelson et al., 2010).* Conjugational plasmid transfer consistently drives the emergence of hyper-virulent or antibiotic-resistant pathogens *(Johnson et al., 2010; Paulsen et al., 2003).* In addition, mutations like SNPs (single nucleotide polymorphisms) or INDELs (INsertion/DELetion) cause small differences between genomes. They are important drivers of bacterial evolution *(Achtman, 2012; Nasser et al., 2014).* Most SNPs will be within protein coding regions in coding-dense genomes like microbes. If the amino acid coded for does not change, it is called a *synonymous* SNP. If it does change, it is called a *non-synonymous* SNP. SNP-typing is starting to be used for epidemiological surveys and multiple phylogeographic analyses at a global scale *(Achtman, 2008; Achtman, 2012).* When mapped to a complete reference genome of a closely related strain, short reads allow the reconstruction of a large proportion of SNPs in the core genome *(Chewapreecha et al.,*

2014; Coll et al., 2014; Nasser et al., 2014). Such SNP calls can potentially allow epidemiological reconstructions of person-to-person transmissions *(Eyre et al., 2013; Harris et al., 2013; Harrison et al., 2014)* and identify infections stemming from a common source *(Zhou et al., 2013).*

1.2.2. Role of *E. coli* in the intestinal microbiota

The microbiota of humans and animals is highly complex, alternating at different parts of the gastrointestinal (GI) tract, and contains approximately 10^{14} bacteria *(Ley et al., 2006a; Savage, 1977; Whitman et al., 1998).* The largest part of the intestinal microbiota consists of four different phyla, namely the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria *(Eckburg and Relman, 2007; Frank et al., 2007; Ley et al., 2006b).* The microbial communities differ in the stomach, small intestine, cecum, and colon, with higher levels of bacterial diversity in the lower segments of the GI tract and higher levels of aerobic and facultative anaerobic bacteria in the stomach and upper small intestine *(Frank et al., 2007; Gu et al., 2013).* These bacteria contribute to the intestinal digestive function, influence epithelial metabolism, stimulate epithelial cell proliferation and gut immunity, and also compete with enteric pathogens. The intestinal microbiota of healthy mammals is robust and composed of diverse groups of bacteria. This stability generally persist over time *(Martinez et al., 2013)* and has a great effect on the host's immunity to infectious diseases *(van der Waiij and Verhoef, 1979).* During an infection with pathogenic microorganisms, the microbiota can have the basic function of blocking the growth of the pathogen and thus they interfere with the infection right from the beginning. This phenomenon has been termed as 'colonization resistance' *(Buffie and Pamer, 2013; van der Waaij et al., 1971).* Colonization resistance could be based on nutrient limitation, release of inhibitory metabolites, production of bactericidal compounds, the competition for binding sites, and other unidentified features of the dense microbial community *(Sekirov and Finlay, 2009; Stecher and Hardt, 2008; Vollaard and Clasener, 1994).* Thus, a high diversity of the bacterial microbiota in the GI tract enhances the robustness of the microbial community and is believed to contribute to a high colonization resistance against pathogens *(Hentges, 1983; Kühn et al., 1993; Kühn et al., 1996).* Besides the direct colonization resistance mediated by microorganisms, recent studies have shown that commensal bacteria can also indirectly control invading pathogens by enhancing host immunity in the intestine *(Buffie et al., 2013; Yurist-Doutsch et al., 2014).* This is known as immune-mediated colonization resistance *(Buffie et al., 2013).* The

microbiota could prime the host's innate *(Chung et al., 2012; Diehl et al., 2013)* and adaptive *(Hand et al., 2012; Lathrop et al., 2011)* immune defenses and prevent pathologic events which would otherwise be caused by virulence factors of pathogens *(Sekirov et al., 2010; Yurist-Doutsch et al., 2014).* Furthermore, it may help to eliminate enteropathogenic bacteria from the gut lumen at the end of an infection *(Endt et al., 2010).* This mechanism is called 'pathogen clearance' and differs significantly from colonization resistance as both the mucosa *(Barthel et al., 2003)* and the microbiota have to recover from pathogen-inflicted disturbance while eliminating the pathogen *(Barman et al., 2008).* As a result of the above mentioned functions it becomes clear that any changes in the stability of the microbiota, especially during early GI tract development, can lead to an overgrowth of native as well as invaded pathogenic bacteria. A consequence resulting from this can be severe diarrhea *(Katouli et al., 1999; Yeoman and White, 2014).* However, different antibiotic and probiotic therapies exist for the restoration of the intestinal microbiota following a disruption *(Gibson et al., 2014).*

After birth, coliforms are one of the first groups of bacteria that colonize the intestine of mammals *(Drasar and Barrow, 1985).* During the suckling period, the population of the coliforms is often not stationary, as several bacteria of different serotypes *(Howe and Linton, 1976),* biotypes *(Hinton et al., 1982; Hinton et al., 1985),* and genotypes *(Nagy et al., 1990)* can exist at any time. Early gut colonization is also critically important to the morphological and immunological development of the GI tract, the development of a functional fermentative environment, and neonatal resistance to pathogens *(Sekirov et al., 2010; Yeoman et al., 2014).*

Although *E. coli* is a facultative anaerobic bacterium, it is the dominant aerobic, intestinal population of Enterobacteriaceae in the microbiota *(Gordon et al., 2003; Hartl and Dykhuizen, 1984; Selander et al., 1987). E. coli* is thought to mainly inhabit the lower GI tract and some studies have observed the distribution of *E. coli* among the different sections of the gut *(Bettelheim et al., 1992; Dixit et al., 2004).* Individual discrepancies in intestinal *E. coli* populations of hosts can be caused by diet, climate, age, sex, and weaning *(Katouli et al., 1995; Katouli et al., 1999).* Weaning in particular initiates a massive change in the intestinal microbiota *(Wu et al., 2007).*

The mucus layer of the mammalian colon is the niche of commensal *E. coli* in the intestine. This bacterium is highly successful in competition for this location. The mechanisms of *E. coli* for ensuring this favorable symbiosis are poorly understood. One suggestion is that *E.*

coli uses its ability to utilize gluconate more efficiently than other resident species. This allows *E. coli* to inhabit a highly specific metabolic niche *(Sweeney et al., 1996)*.

However, acquiring specific virulence genes can lead to highly adapted *E. coli* with an increased ability to adapt to new niches, which could allow them to cause a broad spectrum of diseases. This is believed to provide an evolutionary pathway to pathogenicity *(Chapman et al., 2006; Kaper et al., 2004)*. Virulence genes are frequently encoded on genetic elements that can be mobilized and transferred between different strains, creating novel combinations of virulence factors. Only the most successful combinations persist to become specific pathotypes of *E. coli* that are capable of causing disease in healthy individuals *(Kaper et al., 2004)*.

1.2.3. Virulence of *E. coli* – different pathotypes

Classification of *E. coli* into different pathotypes depends on the content of virulence-associated genes (VAGs), location, and clinical symptoms recorded in the patients. As already mentioned, *E. coli* can be divided into two main groups; namely, intestinal pathogenic and extraintestinal pathogenic *E. coli* (InPEC and ExPEC, see Fig. 1).

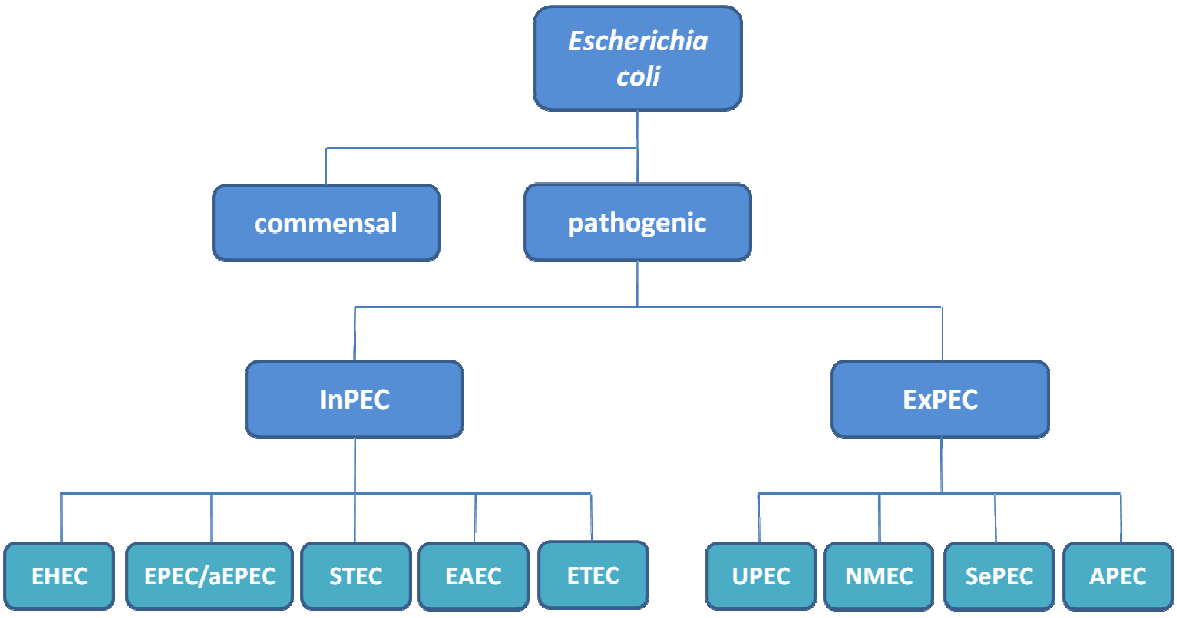

Fig. 1: Overview of *E. coli* pathogens.

InPEC cause different kinds of diarrhea *(Kaper et al., 2004)*. In contrast, ExPEC can cause extraintestinal diseases like urinary tract infections (UTIs) from uropathogenic *E. coli* (UPEC), Sepsis from septicemia-associated (SePEC) and Newborn Meningitis from newborn meningitis-causing (NMEC) *E. coli (Ewers et al., 2009; Ewers et al., 2014;*

Ewers et al., 2007). ExPEC include also avian pathogenic *E. coli* (APEC), which cause systemic infections. This is one of the most relevant diseases caused by ExPEC in animals, leading to significant economical losses in the poultry industry *(Dho-Moulin and Fairbrother, 1999; Ewers et al., 2003).*

Well known and described InPEC pathotypes of human and animals are enterotoxigenic (ETEC), Shiga-toxin producing (STEC), enterohemorrhagic (EHEC), or enteropathogenic *E. coli* (EPEC) *(Nataro et al., 1998).* Intestinal pathotypes are well defined by their possession of distinct combinations of virulence-associated factors determining certain molecular pathways. These pathotypes have a lot of features in common, including virulence gene combinations for attachment and elaboration of e.g. hemolysins and enterotoxins *(Kaper et al., 2004).* However, these virulence genes harbor significant polymorphism and sequence variation in their molecular identities *(Nagy and Fekete, 1999).* Although extraintestinal pathotypes carry specific groups of VAGs, it is not possible to classify them by their VAGs alone. It is also necessary to classify them by clinical symptoms *(Ewers et al., 2007).* A recent study suggested the o454-nlpD region (patterns I-IV) to be of great value for identifying highly virulent strains among the mixed population of *E. coli,* promising to become the basis of a future typing tool for ExPEC and their gut reservoir. In addition, top-ranked VAGs for classification and prediction of pattern III were identified. These data are important for defining ExPEC pathotypes in future *in vivo* assays *(Ewers et al., 2014).* Furthermore, novel fitness factors were continually identified and new insights about UTIs were gained *(Subashchandrabose et al., 2014).*

1.2.4. *E. coli* in pig production

Although *E. coli* are considered to be normal inhabitants of the intestinal tract, several pathotypes cause infections that are one of the main reasons of economic loss in the pig industry *(Katouli et al., 1995; Wieler et al., 2001)*

The composition of the *E. coli* microbiota in pigs is diverse and individual, and virulence gene-carrying *E. coli* strains as well as antimicrobial resistant commensal bacteria are a normal part of intestinal bacterial populations *(Dixit et al., 2004; Gordon et al., 2003; Schierack et al., 2006; Schierack et al., 2007).* However, antimicrobial resistance genes were not associated with a colonization advantage or disadvantage in the intestine of young piglets in the absence of antimicrobial drugs *(Schierack et al., 2009a).*

At birth, the GI tract of the pigs is sterile. Nevertheless, after a short time, the GI tract gets flooded with microorganisms that are able to survive the relatively low gastric pH. The colonizing bacteria are mainly acquired from maternal feces, skin, and teats *(Arbuckle, 1968; Bertschinger et al., 1988)*. Indeed, it has been reported that suckling piglets eat significant amounts of the sow's feces *(Sansom and Gleed, 1981)*. *E. coli* is among these first colonizers of the intestine of piglets *(Drasar et al., 1985)*.

Post-weaning diarrhea (PWD) caused by *E. coli* is one main problem in livestock production and can even lead to death in weaned pigs. Enteric *E. coli* infection manifests as a diarrhea that generally occurs during the first week of post-weaning and often causes lower weight gain. Several factors, such as the stress of weaning, lack of antibodies originating from the sow's milk, and dietary changes contribute to the severity of the diarrhea *(Fairbrother et al., 2005)*.

Enteropathogenic *E. coli* (EPEC), which was first isolated from children, was the first pathotype of *E. coli* to be described. During large outbreaks of infant diarrhea in the United Kingdom in 1945, a group of serologically distinct *E. coli* strains were described. They could be isolated from children with diarrhea but not from healthy children. While large outbreaks of infant diarrhea have disappeared from the industrialized world, EPEC remains an important cause of infant diarrhea in developing countries *(Nataro et al., 1998)*. EPEC are also present in pigs and are implicated in PWD that can cause attaching and effacing (AE) lesions. They are therefore called attaching and effacing *E. coli* (AEEC) *(An et al., 2000; Janke et al., 1989; Zhu et al., 1994)*. The ability to induce this AE histopathology is encoded by genes on a 35-kb pathogenicity island (PAI) called the locus of enterocyte effacement (LEE) *(McDaniel et al., 1995)*. Identification of porcine EPEC (PEPEC) is difficult and veterinary diagnostic laboratories do not routinely attempt to identify this pathotype of *E. coli*. Investigations have been carried out revealing that EPEC appears to be involved in about 6% of cases of PWD *(Fairbrother, 1999)*.

ETEC is the most prevalent pathogenic *E. coli* in weaned piglets. PWD is also primarily caused by this pathotype. ETEC is characterized by production of adhesins like fimbriae F4 (K88), F5 (K99), F6 (987P), F17 (Fy/Att25), F41, and F18 that occur in pigs and calves and mediate bacterial adherence to the intestine, as well as enterotoxins (heat-labile [LT] and/or heat-stable [ST]), which cause secretory diarrhea in new born and weaned piglets *(Frydendahl, 2002; Schierack et al., 2006)*.

Shiga toxin–encoding *E. coli* (STEC) strains encoding the Shiga toxin type 2e (Stx2e) represent the causative agents of edema disease in weaning piglets *(MacLeod et al.,*

1991). The characteristic genes of edema disease causing *E. coli* (EDEC) are *stx*2e (Shiga toxin 2e) and *fedA* (F 18) *(Fairbrother et al., 2005; Schierack et al., 2006).*
In addition, some ETEC strains also harbor the Stx2e genes and therefore may be capable of causing symptoms of edema disease and those of diarrhea in the same animal (STEC/ETEC) *(Barth et al., 2007).* Porcine STEC and many porcine ETEC strains express the F18 fimbriae, which facilitates bacterial colonization of the mucosal surface of the intestine *(Fairbrother and Gyles, 2006).* Other porcine STEC and STEC/ETEC strains can express other cytoadhesive fimbriae or nonfimbrial adhesins, in addition to the F18 fimbria and in particular F4 or F5 fimbriae or the "adhesin involved in diffuse adherence" (AIDA) *(Barth et al., 2007; DebRoy et al., 2009; Gyles and Fairbrother, 2010; Niewerth et al., 2001)*. While the AIDA occurs frequently in both pathovars, F4 or F5 fimbriae are rare in those strains *(Gyles, 2007).* A preventing strategy is to vaccinate the piglets. In particular it was shown that pigs vaccinated with one F18 fimbrial subtype (F18ab, F18ac) were not or were only partially protected during challenge experiments with ETEC strains expressing the other fimbrial subtype *(Bertschinger et al., 2000; Sarrazin and Bertschinger, 1997).* A fimbrial vaccine directed against porcine STEC/ETEC infection in Germany would have to cover both subtypes of F18 fimbriae in order to provide significant protection *(Barth et al., 2011).*

Schierack et al. (2008) found ExPEC colonizing the gut of healthy pigs, assuming the intestine of pigs as reservoir for ExPEC without any symptoms inside the intestine. However, they cause extraintestinal diseases like urinary tract infection or sepsis outside the intestine *(Ewers et al., 2007).* Typical virulence associated genes (VAGs) for ExPEC are a number of serum resistance, adhesion, and iron acquisition genes *(Ewers et al., 2007).* Until now there is no fixed definition for classifying ExPEC. The different ExPEC types are only categorized according to their pathogenicity. However, a new, promising typing method using the o454-nlpD region has been developed in a recent study *(Ewers et al., 2014).*

1.3. Feed supplements – Probiotics and Zinc

Before 2006 antibiotics were widely used in the European Union as feed supplements for growth promotion and prevention of infections in farm animals. Because of the rising incidence of multi-resistant microorganisms in animal production over the last decades

(Ewers et al., 2012; Jensen, 2006; Villanueva, 2012), the European Union prohibited antibiotic growth promoters as feed supplements in animal breeding *(EC, 2005)*. In Denmark, pig farmers voluntarily abolished all use of antimicrobial growth promoters in pig feed, starting in the year 2000. As a consequence several negative effects arose, such as compromised thriving, poorer growth performance, and an increased incidence of diarrhea among piglets within the first weeks after weaning *(Hojberg et al., 2005)*. Thus, there was an urgent need to find and evaluate substances to be used as alternatives to antimicrobial growth promoters for use in livestock farming. So far different alternatives have been investigated for their growth promoting effect and health benefits. The most commonly used alternatives are pre- and pro-biotics or divalent metal cations, such as zinc and copper *(Heo et al., 2013; Lalles et al., 2007)*.

The following sections will provide more detailed information on the feed supplements *E. faecium* and zinc, especially in pig breeding.

1.3.1. Probiotics

1.3.1.1. Use of Probiotics in animal housing

Various studies investigate the important role that probiotic bacteria play in the modulation of immunological and gastrointestinal function in pigs *(Collado et al., 2007; Franz et al., 2011; Scharek et al., 2005; Underdahl et al., 1982)*. It has been shown that by directly competing with intestinal pathogens, probiotics have a protective effect. Probiotics are defined as living bacteria that, when administered in adequate amounts, confer a health benefit on the host *(FAO/WHO, 2002)*. The mechanisms of action are currently not fully understood.

Probiotic bacteria are commonly administered in their viable forms. Studies have demonstrated that the viability of bacteria enhances the probiotic effect *(Panigrahi et al., 2005)*. This fact suggests that probiotic bacteria should survive the passage through the gastrointestinal tract and persist in the intestine *(Galdeano and Perdigon, 2004)*. Survival and persistence in the gut requires tolerance to the environment as well as competition with the already existing host microbiota *(Dunne et al., 2001; Saarela et al., 2000)*. The number of probiotic bacteria declines within a few days or weeks after termination of supplementing, requiring continued feeding of probiotics to maintain the positive effects *(Kleta et al., 2006)*.

Most probiotic microorganisms are Gram-positive, with lactobacilli or Bifidobacteria being the main species used as treatments for intestinal dysfunctions in humans *(Marco et al., 2006)*. They were originally isolated from the human gastrointestinal tract. Enterococci, which also occur in this ecosystem, are used as probiotics to a much lesser extent, especially in food for humans. Established enterococcal probiotics include *E. faecium* SF68® (NCIMB 10415, produced by Cerbios-Pharma SA, Barbengo, Switzerland), especially used in pig production, and *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany). However, some Gram-negatives are also used as probiotics, like *Escherichia coli* Nissle 1917 (EcN) *(Nissle, 1959)*.

1.3.1.2. *Enterococcus faecium* NCIMB 10415 in pig production

Until the 1980s all Gram-positive cocci were described as streptococci, then they were reclassified as the genera *Streptococcus*, *Lactococcus*, and *Enterococcus (Devriese et al., 1993; Devriese et al., 1995; Schleifer et al., 1984)*. The genus *Enterococcus* comprises the third-largest of lactic acid bacteria (LAB) after the genera *Lactobacillus* and *Streptococcus*. Currently 37 species are described *(Devriese et al., 2003; Franz and Holzapfel, 2006)* (http://www.bacterio.net). Members of the genus *Enterococcus* can be found in the gastrointestinal tract of humans and animals. They are also found in other habitats, such as in the soil, on plants, and in the intestines of insects and birds. The strain *E. faecium* NCIMB 10415 (SF68) seems to be the best studied probiotic bacteria for use in animals. This strain has a robust nature and is able to survive and properly grow under intestinal conditions, which is an important ability for its probiotic activity *(Franz et al., 2011)*. A study with piglets showed that *E. faecium* survives the stomach passage *(Spieler and Männer, 1996)*. In addition, the strain could be distinguished from other enterococci in the gastrointestinal tract of piglets *(Vahjen et al., 2007)*.

Previous studies have already demonstrated positive effects of *E. faecium* on the microbiota and the immune system in piglets, especially in the weaning period. Zeyner and Boldt (2006) showed that the daily oral intake of *E. faecium* from birth until weaning reduces the number of piglets with diarrhea. This resulted in a higher weight gain of the piglets *(Zeyner and Boldt, 2006)*. A study with *E. coli* infected gnotobiotic piglets indicated that the probiotic strain has a positive influence on the health of piglets. In addition to enhanced weight gain, the pigs had less severe diarrhea. Animals without *E. faecium* supplementation developed severe diarrhea and lost weight, and some also died

(Underdahl et al., 1982). E. coli and the probiotic were equally distributed in the jejunum of all groups. Thus, an abundant colonization with *E. faecium* could be demonstrated. The authors speculated that *E. faecium* reduces the toxic effect of *E. coli (Underdahl et al., 1982)*, without determining any detailed mechanism.

Studies of the "DFG Forschergruppe 483" (precursor of the CRC 852) showed that *E. faecium* NCIMB 10415 changed the swine intestinal microbiota by reducing natural infection by *Chlamydia* and pathogenic intestinal *E. coli* serotypes *(Pollmann et al., 2005; Scharek et al., 2005).* However, the total composition of Enterobacteriaceae was not changed significantly, indicating a specific effect against pathogens *(Schierack et al., 2007).*

Recent studies of the CRC 852 have contributed to a better understanding of the probiotic effect of *E. faecium* NCIMB 10415. One study suggests that *E. faecium* did not clearly influence physiological variables associated with digestive function. The main factor for determining function and morphology of the jejunal tissue seems to be the age of piglets. This could reflect both maturation and adaptation to constant feeding *(Martin et al., 2012).* The results of another study indicated that probiotic bacteria could have effects on virus shedding in naturally infected pigs, depending on the virus type. These effects seem to be caused by immunological changes. However, the distinct mechanisms of action remain to be elucidated *(Kreuzer et al., 2012b). E. faecium* increased the absorptive and secretory capacity of jejunal mucosa and improved the intestinal barrier functions. These effects could protect against the colonization of pathogenic bacteria and infiltration of toxins, and thereby prevent diarrhea. Additionally, an enhanced uptake of nutrients could follow as a result of more liquid ingesta and increased absorption. As probiotic feeding has been shown to change cytokine expression in various tissues, they could be potential mediators of the probiotic effects *(Klingspor et al., 2013).*

In a challenge trial with a control and probiotic group that were both infected with swine influenza virus (H3N2) *E. faecium* was shown to have a positive effect on the humoral immune response after vaccination and recovery. However, there was no effect on virus shedding or lung pathology *(Wang et al., 2014).*

In a challenge trial with *Salmonella* Typhimurium DT104 the feeding of *E. faecium* NCIMB 10415 resulted in an increased infection in piglets, but nevertheless also to an enhanced production of specific antibodies against *Salmonella (Szabo et al., 2009).* A reduction of certain T-cells, which led to a severe *Salmonella* infection, could also be observed *(Mafamane et al., 2011).* Siepert et al. 2014 suggested that feeding of pre-

weaning piglets with *E. faecium* influences intestinal immune-associated gene expression, which gets worse in post-weaning piglets when the animals take up a higher concentration of the probiotic with the diet. The post-weaning reductions in gene expression might delay the host response to infections and give pathogenic bacteria a "window of opportunity," leading to increased bacterial loads and shedding *(Siepert et al., 2014)*. These data verify that *E. faecium* could have an immunosuppressive effect on the host immune system, which might reduce inflammation.

1.3.2. Zinc

1.3.2.1. Zinc as trace element in bacteria

Bivalent metal cations like zinc are essential trace elements for pro - and eukaryotic cell functions. They are essential components of several enzyme systems *(Riordan, 1976)*, such as alkaline phosphatases and terminal oxidases *(Blencowe and Morby, 2003)*. Zinc is contained in very low concentrations in natural feed. It is essential for various physiological functions and serves as a component e.g. for DNA and RNA-polymerases *(Holzel et al., 2012)*. Higher concentrations of zinc are toxic for bacteria. Therefore the secretion and excretion of zinc in prokaryotes is strictly regulated *(Nies, 1999)*. Under zinc deficiency conditions the zinc uptake in *E. coli* is enabled by a high affinity transport system, called ZnuABC *(Patzer and Hantke, 1998)*. The efflux of zinc is regulated by two different systems, which also play a role in zinc resistance *(Grass et al., 2002)*. In *E. coli* these systems are named ZntA, a metal transporting ATPase *(Rensing et al., 1997; Sharma et al., 2000)*, and ZitB, a transport protein of the cation diffusion facilitator (CDF) family *(Anton et al., 2004; Grass et al., 2002)*.

1.3.2.2. Effect of zinc on the microbiota of pigs

Within the last years, the use of zinc in livestock farming has become more important and remains popular as a feed supplement to prevent diarrhea in weaning piglets. However, Zinc's mechanisms of action in pigs remain only poorly defined.

Particularly zinc in the form of zinc oxide (ZnO) has prevailed as a feed supplement in commercial pig farming. Other metals, such as copper, did not show such strong effects *(Hojberg et al., 2005)*. Zinc's antibacterial effect has already been assumed for a long time. Samples taken from the jejunum of pigs were cultivated with 100 ppm zinc, which reduced the number of *E. coli*. This reduction could not be observed *in vivo* by feeding 1,350 ppm

zinc to animals *(Jensen, 1987)*. However, a concentration of 2,500 ppm ZnO added to the diet showed a prophylactic effect comparable to that of the antibiotic olaquindox *(Holmgren, 1994)*. The basic physiological need for zinc in pigs implies a need for a dietary supplement, typically in the range of 50 to 100 ppm zinc *(Jondreville et al., 2002; Revy et al., 2003)*. Zinc deficiency in pigs is mostly known as a cause of parakeratosis *(Tucker and Salmon, 1955)*, which mainly appears in pigs from 5-16 weeks of age. Up to 50% of pigs may be affected. Major signs are limited to the skin, where gross thickening and roughening occurs over the whole body. It can start with small light brown spots or papules on the legs and abdomen. In young pigs it can look like greasy pig disease. However, high amounts of zinc can be toxic for animals as well as for the environment. The tolerated amount of zinc as feed supplement depends on the choice of the chemical bond. Brink et al. (1959) showed that 2,000 ppm zinc carbonate fed to weaned piglets over a period of four weeks caused symptoms of toxicity and lower feed intake and performance of piglets *(Brink et al., 1959)*. In contrast, no evidence for toxicity was detected for the same concentration of zinc fed in the form of zinc oxide, which result can be explained by its different solubility *(Damgaard Poulsen, 1989; Hsu and Anthony, 1975)*.

Many recently published studies are concerned with the influence of zinc on bacteria and their hosts. Certain concentrations of zinc can lead to positive effects, such as growth promotion and lower numbers of bacterial populations in the intestine, e.g. lactobacilli *(Hojberg et al., 2005; Vahjen et al., 2011)*.

The direct influence of pharmacological zinc concentrations on piglets could affect the gene expression responsible for glutathione metabolism and apoptosis *(Wang et al., 2009)*. In addition, increased gastric ghrelin secretion enhancing the feed intake *(Yin et al., 2009)* or the production of digestive enzymes can occur *(Hedemann et al., 2006)*. As epithelial cells are the first barrier to pathogen invasion, zinc may play a particular role in resistance to infections *(Chandra and Au, 1980)*. Additionally, there are studies which imply that zinc is a reducing factor for virulence in bacteria. Zinc can be toxic or inhibit many strains of *Staphylococcus aureus* at concentrations over 0.001 mol/l *(Walsh et al., 1994)*. Endotoxins from *Salmonella enterica* Typhi and hemolysins from *Aeromonas hydrophila* were inhibited by inorganic zinc salts *(Filteau and Tomkins, 1994; WHO, 1987)*. Fenwick et al. 1990 demonstrated that zinc-deficient rats were unable to distribute the intestinal load of *Trichinella spiralis*, compared to pair-fed and *ad libitum* zinc fed animals *(Fenwick et al., 1990)*.

In addition to its effects on the host, increased dietary zinc affects the diversity of the intestinal microbial community. For instance, lactobacilli colony counts were reduced, while coliform colony counts were increased by using 2,500 ppm of dietary zinc in a study with piglets *(Hojberg et al., 2005)*. This corresponds to a challenge study with post-weaning piglets, in which an increased shedding of the inoculated pathogenic *E. coli* strain was shown *(Mores et al., 1998)*. Reduced anaerobic and lactic acid bacteria colony counts were also reported. However, no effect on *E. coli* could be determined *(Broom et al., 2006)*. Thus, the frequently observed reduction of post-weaning diarrhea in zinc-supplemented piglets might not be related to a general reduction of *E. coli (Fairbrother et al., 2005),* and the impact of zinc on the diversity of the coliform community may be more important *(Katouli et al., 1999)*.

Vahjen et al. (2012) described a higher diversity of Enterobacteriaceae in the intestine of pigs under high zinc supplementation. This effect could help to stabilize the intestinal microbiota in the post-weaning period *(Vahjen et al., 2012)*. Another study showed that increased levels of dietary ZnO had strong and dose-dependent effects on the ileal bacterial community composition and activity, suggesting taxonomic variation in metabolic response to ZnO *(Pieper et al., 2012)*. *Campylobacter (C.) coli* showed higher sensitivity against ZnO. The addition of high amounts of zinc reduced the shedding of *C. coli*. Initial data indicate that *C. coli* reacts to zinc with an oxidative stress response *(Bratz et al., 2013)*. Some amounts of zinc (2,500 ppm ZnO) change the ileal bacterial composition on the species level *(Vahjen et al., 2011)*, in particular by reducing the predominant lactobacilli.

High zinc concentrations also seem to have a positive influence on different virus infections. It was demonstrated that zinc can provide a general protection for the intestinal tract, as the systemic humoral immune response is stimulated against transmissible gastroenteritis-virus (TGEV) infection. A similar positive effect was shown after vaccination and recovery from a swine influenza virus (SIV) infection *(Chai et al., 2014; Wang et al., 2014)*.

1.3.2.3. Potential influence of zinc on *E. coli* as member of the intestine

Recent literature suggests that zinc may promote bacterial diversity and the spread of antimicrobial resistance, in addition to reducing the virulence of assigned bacteria, such as that of some types of *E. coli (Crane et al., 2011; Crane et al., 2007; Holzel et al., 2012; Vahjen et al., 2012)*.

A diet containing high zinc concentrations in piglets led to the detection of more bacterial species *in vivo*, indicating a greater bacterial diversity. Additionally, more enterobacterial species could be detected in the animals *(Vahjen et al., 2012)*. An increase of Enterobacteriaceae may be due to a better colonization potential of this group of bacteria. This could lead to stronger competition between Enterobacteriaceae and the reduced occurrence of pathogenic *E. coli* strains, which are the main cause of diarrhea after weaning *(Vahjen et al., 2011)*.

Moreover, it was demonstrated that zinc reduces the secretion of enteropathogenic *E. coli* (EPEC) virulence factors *in vitro (Crane et al., 2007)* and inhibits adherence in a cell culture model with HeLa cells as well as expression of EspA and Stx in STEC strains *(Crane et al., 2011)*.

An *in vitro* model of ETEC infection with IPEC-J2 cells and supplementation of zinc confirmed an increased expression of many innate immune response genes (NF-κB targets) and several stress response genes in response to ETEC exposure. The expression of genes involved in innate immune response was reduced when cells were simultaneously exposed to ZnO. The treatment with ZnO could inhibit the induction of NF-κB in response to pathogens, probably through up-regulated heat shock proteins. A consequent down-regulation in the inflammatory response *in vivo* would reduce further pathogen invasion, continuing normal gut function and growth *(Sargeant et al., 2011)*.

Although zinc was initially used as substitute for antibiotics, there are several publications in favor of the idea that zinc might promote the spread of antimicrobial resistance via different mechanisms, such as physiological (cross-resistance) or genetic coupling (co-resistance) *(Lee et al., 2005; Nishino et al., 2007)* or direct interactions. It has already been shown that zinc affects the stability of ampicillin *(Mukherjee and Ghosh, 1995)*. Zinc-dependent beta-lactamases have been described *(Cooper et al., 1993)*. Zinc may influence bacterial conjugation rates *(Ou, 1973; Ou and Anderson, 1972)* and affects the expression of genes in *E. coli* K12 including: antibiotic resistance efflux-pumps, metal ion efflux systems, adhesins, and flagellae *(Lee et al., 2005)*. However, as the work by Lee et al. only investigated *E. coli* K12, and as outlined above, *E. coli* exists in a huge genomic diversity, the reaction of porcine wild-type isolates of *E. coli* is basically unstudied. The role of zinc as a feed supplement therefore needs to be critically evaluated.

1.4. Aims of the thesis

The overall aim of this thesis was to characterize the dynamics of the porcine intestinal *E. coli* population under the influence of the probiotic *Enterococcus faecium* strain NCIMB 10415 and the feed additive zinc in piglets with two animal trials. The main hypothesis was that both supplements influence the *E. coli* population in the intestine in pigs. The bivalent metal cation zinc may have a direct effect on the *E. coli* diversity and antimicrobial resistance, while *E. faecium* should have a positive effect on pathogenic *E. coli* and the health of the pigs. Positive effects of *E. faecium* were already shown in previous preliminary studies *(Pollmann et al., 2005; Scharek et al., 2005; Taras et al., 2006; Vahjen et al., 2011)*

The most important steps to reach the aims were:

i. Investigation of the effect of zinc supplementation on the *E. coli* population in the intestine of pigs (Study 1)
ii. Identification of the effect of *E. faecium* strain NCIMB 10415 feed supplementation on the intestinal *E. coli* population in swine (Study 2).

1.5. Design of the thesis

To provide a better understanding and overview of the different parts of the project, the materials, methods, and groups of the studies are shown in Tables 1 and 2. The project was subdivided into two major sections. For identification and characterization of the *E. coli* population in the swine gut with *E. faecium* and zinc as feed additives two different animal trials were performed.

i. The zinc trial included 36 piglets (Study 1, Fig.1, Tab.1)
ii. 24 piglets were used for the *E. faecium* trial (Study 2, Fig.2, Tab.2).

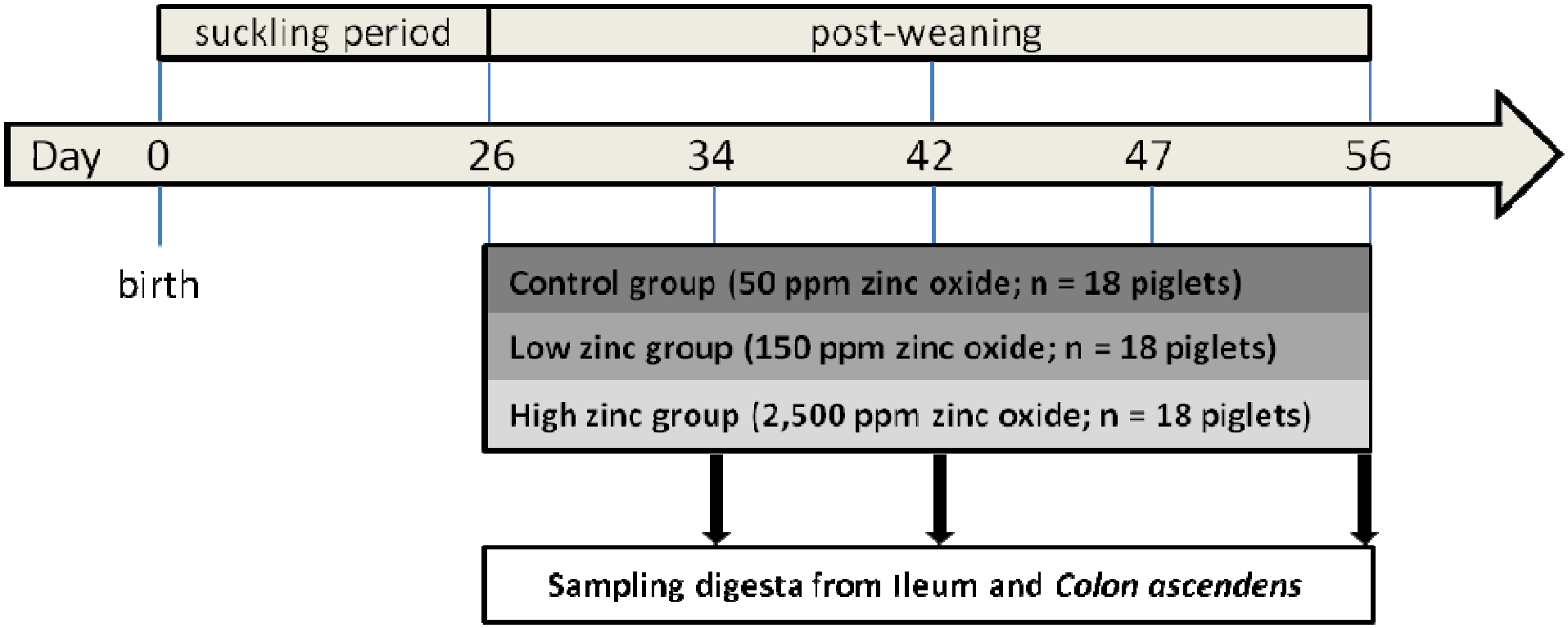

Fig. 2: Scheme of the animal trial with feeding time points of zinc supplementation. Day 0 is the time of birth. From day 26 piglets were supplemented with different zinc concentrations. Weaning started at the same time point as zinc supplementation (Details see Tab. 1).

Tab. 1: Design of zinc feeding trial with 36 young piglets

Group	Age in days (d)	Samples	Agar Plates (2 plates each dilution)	Sample dilutions	Isolation	Methods for characterization
Control group (50 ppm[a] zinc)	32d ± 1d (n = 6) 41d ± 1d (n = 6) 53d ± 1d (n = 6)	Digesta Ileum Digesta Colon as.	Chrom[b] Sheep blood Gassner Chrom + Amp[c] Chrom + Tetra[d] Chrom + Strep[e] Chrom + Cefo[f]	10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-1} - 10^{-3} 10^{0} - 10^{-2} 10^{0} - 10^{-2} 10^{0} - 10^{-2}	Picking 20-24 colonies per sample/animal	PFGE[g], VAG-PCR[h], MLST[i], ADD[j], MIC[k]
Low zinc group (150 ppm[a] zinc)	32d ± 1d (n = 6) 41d ± 1d (n = 6) 53d ± 1d (n = 6)	Digesta Ileum Digesta Colon as.	Chrom[b] Sheep blood Gassner Chrom + Amp[c] Chrom + Tetra[d] Chrom + Strep[e] Chrom + Cefo[f]	10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-1} - 10^{-3} 10^{0} - 10^{-2} 10^{0} - 10^{-2} 10^{0} - 10^{-2}	Picking 20-24 colonies per sample/animal	PFGE[g], ADD[j], MIC[k]
High zinc group (2500 ppm[a] zinc)	32d ± 1d (n = 6) 41d ± 1d (n = 6) 53d ± 1d (n = 6)	Digesta Ileum Digesta Colon as.	Chrom[b] Sheep blood Gassner Chrom + Amp[c] Chrom + Tetra[d] Chrom + Strep[e] Chrom + Cefo[f]	10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-1} - 10^{-3} 10^{0} - 10^{-2} 10^{0} - 10^{-2} 10^{0} - 10^{-2}	Picking 20-24 colonies per sample/animal	PFGE[g], VAG-PCR[h], MLST[i], ADD[j], MIC[k]

[a]ppm, parts per million
[b]Chrom, CHROMagar®
[c]Amp, ampicillin
[d]Tetra, tetracyclin
[e]Strep, streptomycin
[f]Cefo, cefotaxim
[g]PFGE, pulse-field gel electrophoresis
[h]VAG-PCR, virulence-associated-genes PCR
[i]MLST, multi-locus sequence typing
[j]ADD, agar disk diffusion testing
[k]MIC, minimal inhibitory concentration

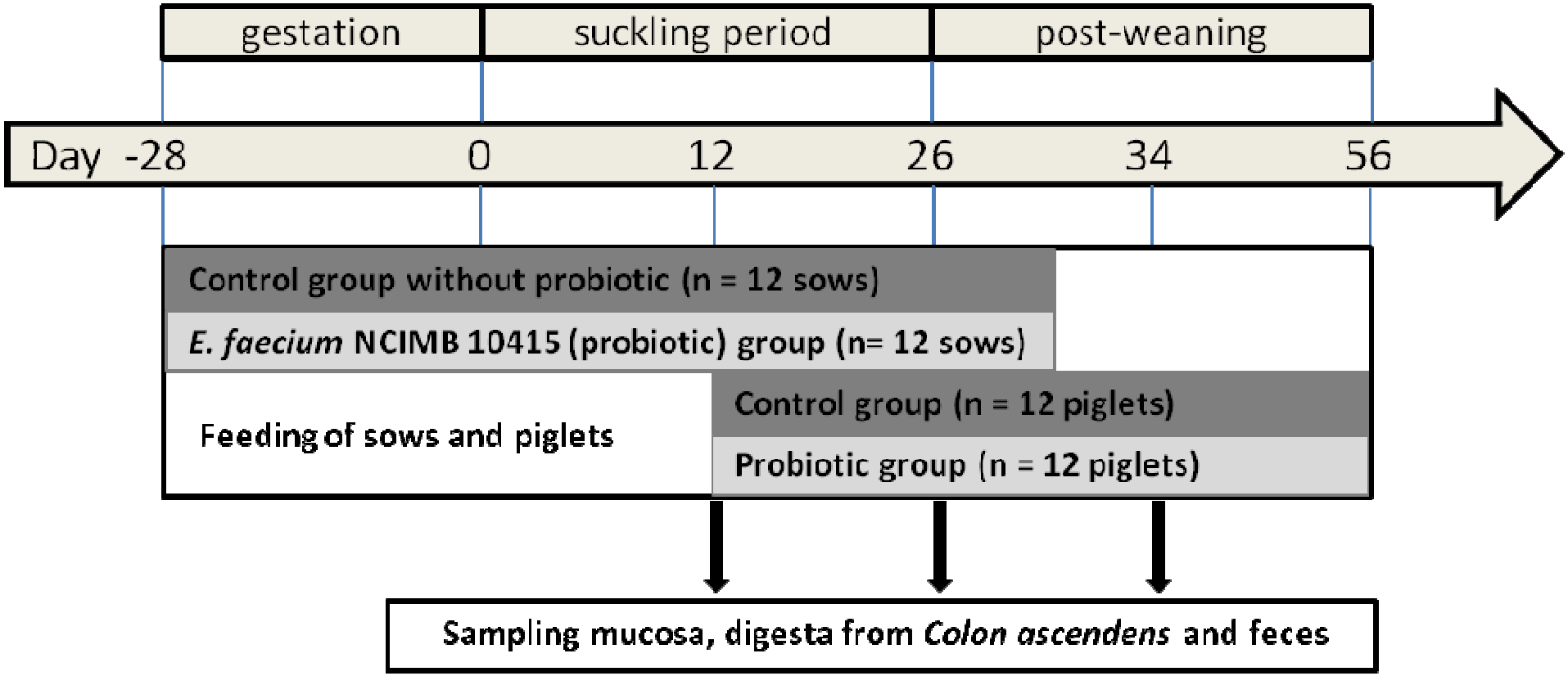

Fig. 3: Scheme of the animal trial with feeding time points of probiotic *E. faecium* NCIMB 10415 supplementation. Day 0 is the time of birth, negative numbers indicate days of gestation, and other days indicate the dates of killing the animals for sampling. From day 12, in addition to suckling, pre-starter feeding of the piglets commenced with or without supplementation of the probiotic *E. faecium* NCIMB 10415. Weaning was at day 26 (Details for sampling see Tab.2).

Tab. 2: Design of *Enterococcus faecium* NCIMB 10415 trial with 24 young piglets

Group	Age in days (d)	Samples	Agar Plates (2 plates each dilution)	Sample dilutions	Isolation	Methods for characterization
Control group (non-supplemented)	12d ± 1d (n = 4) 26d ± 1d (n = 4) 33d ± 1d (n = 4)	Mucosa (Colon as.) Digesta (Colon as.) Feces	Chrom[a] Sheep blood Gassner Chrom + Amp[b] Chrom + Tetra[c] Chrom + Strep[d]	10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-1} - 10^{-3} 10^{0} - 10^{-2} 10^{0} - 10^{-2}	Picking 20-24 colonies per sample/animal	PFGE[e], VAG-PCR[f], MLST[g]
Probiotic group (*E. faecium*-supplemented)	12d ± 1d (n = 4) 26d ± 1d (n = 4) 33d ± 1d (n = 4)	Mucosa (Colon as.) Digesta (Colon as.) Feces	Chrom[a] Sheep blood Gassner Chrom + Amp[b] Chrom + Tetra[c] Chrom + Strep[d]	10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-3} - 10^{-5} 10^{-1} - 10^{-3} 10^{0} - 10^{-2} 10^{0} - 10^{-2}	Picking 20-24 colonies per sample/animal	PFGE[e], VAG-PCR[f], MLST[g]

[a]Chrom, CHROMagar™
[b]Amp, ampicillin
[c]Tetra, tetracyclin
[d]Strep, streptomycin
[e]PFGE, pulse-field gel electrophoresis
[f]VAG-PCR, virulence-associated-genes PCR
[g]MLST, multi-locus sequence typing

2. Publications and disclosure of personal contributions

2.1. Publication 1

2.1.1. Publication

Bednorz C, Oelgeschläger K, Kinnemann B, Hartmann S, Neumann K, Pieper R, Bethe A, Semmler T, Tedin K, Schierack P, Wieler LH, Guenther S. (2013):

The broader context of antibiotic resistance: zinc feed supplementation of piglets increases the proportion of multi-resistant *Escherichia coli* in vivo.

Int J Med Microbiol. 2013 Aug; 303(6-7): 396-403. doi: 10.1016/j.ijmm.2013.06.004. Epub 2013 Jun 14. http://dx.doi.org/10.1016/j.ijmm.2013.06.004

2.1.2. Contribution

I designed, structured, and prepared the manuscript and the experiments described in the publication. In addition, I discussed and interpreted the results. The final manuscript version was prepared by me after having discussed it with all the authors mentioned on the manuscript. KO helped with preparing for the animal trials, plating the samples, and picking the colonies. Further, she helped me with the experiments, such as PFGE, VAG – PCR, MIC, Plasmid profile analysis and Southern Blot. BK helped with the experiments, such as PFGE and MLST. SH drafted and critically revised the manuscript. KN advised and performed the statistical analyses. RP was responsible for the set up of the animal trials and he drafted and critically revised the manuscript. AB drafted and critically revised the manuscript. TS helped with the analysis of sequence data of the MLST and with the figures. TK drafted and critically revised the manuscript. PS was responsible for the concept of the project, drafted and critically revised the manuscript. LHW drafted and critically revised the manuscript for important intellectual content and gave final approval of the version to be published. Additionally, he took part in writing parts of the manuscript and in the structural arrangement of the paragraphs, and he was responsible for the concept of the project. SG took part in writing parts of the manuscript, drafted and critically revised the manuscript for important intellectual content and gave final approval of the version to be published. In addition, he took part in adjusting the text.

All authors read and approved the final manuscript.

2.1.3. Summary of Publication 1

Following the Europe-wide ban of antimicrobial growth promoters, feed supplementation with zinc has increased in livestock breeding. In addition to possible beneficial effects on animal health, feed supplementation with heavy metals is known to influence the gut microbiota and may promote the spread of antimicrobial resistance via co- or cross-selection or other currently unknown mechanisms. As *Escherichia coli* is among the most important pathogens in pig production and often displays multi-resistant phenotypes, we set out to investigate the influence of zinc feed additives on the composition of the *E. coli* populations *in vivo* focusing on phylogenetic diversity and antimicrobial resistance.

In a piglet feeding trial, *E. coli* were isolated from ileum and colon digesta of high dose zinc-supplemented (2,500 ppm) and background dose (50 ppm) piglets (control group). The *E. coli* population was characterized via pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) for the determination of the phylogenetic background. Phenotypic resistance screening via agar disk diffusion and minimum inhibitory concentration testing was followed by detection of resistance genes for selected clones.

We observed a higher diversity of *E. coli* clones in animals supplemented with zinc compared to the background control group. For further analysis the clones from both feeding groups were divided into three groups: clones appearing only in the control group (control only), clones appearing only in the zinc group (zinc only), and clones appearing in both groups (shared). The proportion of multi-resistant *E. coli* significantly increased in the zinc group only compared to the control group only (18.6% vs. 0%). The shared group harbored 13.2% multi-resistant *E. coli*. This group consists of clones that could be isolated from both feeding groups.

For several subclones present both in the feeding and the control group, we detected up to three additional phenotypic and genotypic resistances in the subclones isolated from the zinc feeding group. Characterization of these subclones suggests a higher proportion in antimicrobial resistance due for example to influences on plasmid uptake by zinc supplementation, which questions the reasonableness of zinc feed additives resulting from the ban of antimicrobial growth promoters.

2.2. Publication 2

2.2.1. Publication

Bednorz C, Guenther S, Oelgeschläger K, Kinnemann B, Pieper R, Hartmann S, Tedin K, Semmler T, Neumann K, Schierack P, Bethe A, Wieler LH. (2013):

Feeding the probiotic *Enterococcus faecium* strain NCIMB 10415 to piglets specifically reduces the number of *Escherichia coli* pathotypes that adhere to the gut mucosa.

Appl Environ Microbiol. 2013 Dec; 79(24):7896-904. doi: 10.1128/AEM.03138-13. Epub 2013 Oct 11. http://dx.doi.org/10.1128/AEM.03138-13

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Feeding the Probiotic *Enterococcus faecium* Strain NCIMB 10415 to Piglets Specifically Reduces the Number of *Escherichia coli* Pathotypes That Adhere to the Gut Mucosa

Carmen Bednorz,[a,f] Sebastian Guenther,[a] Kathrin Oelgeschläger,[a] Bianca Kinnemann,[a] Robert Pieper,[b] Susanne Hartmann,[c] Karsten Tedin,[a] Torsten Semmler,[a] Konrad Neumann,[d] Peter Schierack,[e] Astrid Bethe,[a] Lothar H. Wieler[a]

Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany[a]; Institute for Animal Nutrition, Freie Universität Berlin, Berlin, Germany[b]; Institute of Immunology, Freie Universität Berlin, Berlin, Germany[c]; Department of Biometry and Clinical Epidemiology, Charité Universitätsmedizin Berlin, Berlin, Germany[d]; Faculty of Natural Sciences, University of Applied Sciences, Senftenberg, Germany[e]; Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Berlin, Germany[f]

Feed supplementation with the probiotic *Enterococcus faecium* for piglets has been found to reduce pathogenic gut microorganisms. Since *Escherichia coli* is among the most important pathogens in pig production, we performed comprehensive analyses to gain further insight into the influence of *E. faecium* NCIMB 10415 on porcine intestinal *E. coli*. A total of 1,436 *E. coli* strains were isolated from three intestinal habitats (mucosa, digesta, and feces) of probiotic-supplemented and nonsupplemented (control) piglets. *E. coli* bacteria were characterized via pulsed-field gel electrophoresis (PFGE) for clonal analysis. The high diversity of *E. coli* was reflected by 168 clones. Multilocus sequence typing (MLST) was used to determine the phylogenetic backgrounds, revealing 79 sequence types (STs). Pathotypes of *E. coli* were further defined using multiplex PCR for virulence-associated genes. While these analyses discerned only a few significant differences in the *E. coli* population between the feeding groups, analyses distinguishing clones that were uniquely isolated in either the probiotic group only, the control group only, or both groups (shared group) revealed clear effects at the habitat level. Interestingly, extraintestinal pathogenic *E. coli* (ExPEC)-typical clones adhering to the mucosa were significantly reduced in the probiotic group. Our data show a minor influence of *E. faecium* on the overall population of *E. coli* in healthy piglets. In contrast, this probiotic has a profound effect on mucosa-adherent *E. coli*. This finding further substantiates a specific effect of *E. faecium* strain NCIMB 10415 in piglets against pathogenic *E. coli* in the intestine. In addition, these data question the relevance of data based on sampling fecal *E. coli* only.

In-feed antibiotics have been used in livestock for decades to decrease the risk of infectious diseases and promote growth performance (1, 2). However, in 2006, the use of antibiotics as growth promoters was prohibited in the European Union (3); instead, feed supplements, such as prebiotics, probiotics, or cations, like zinc, are used. In pigs, probiotics, such as *Enterococcus faecium* or *Bacillus cereus* variant Toyoi, are commonly used, based on prior reports of positive effects against microbial infections (4–6, 8).

In previous studies, we investigated the probiotic *E. faecium* strain NCIMB 10415 as a feed supplement in piglets. We observed that *E. faecium* did not change the general swine intestinal microbiota (9) but showed specific effects reducing natural infections by *Chlamydia* spp. and pathogenic intestinal *Escherichia coli* serotypes (10, 11).

E. coli is a member of the gastrointestinal autochthonous microbiota of pigs and contributes to the maintenance of the microbial gut balance (12). However, in addition to commensal strains, pathogenic strains causing intestinal or extraintestinal diseases are a great health concern for both humans and animals (13, 14). Intestinal pathogenic (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC) strains are classified into certain pathotypes according to possession of virulence-associated genes (VAGs). Well-known InPEC pathotypes are enterotoxigenic (ETEC), enteropathogenic (EPEC), or Shiga-toxin producing (STEC), while typical ExPEC ones are uropathogenic (UPEC), newborn meningitis-causing (NMEC), septicemia-associated (SePEC), and avian-pathogenic (APEC) *E. coli* (13–15).

Porcine intestinal *E. coli* populations have been described as being highly individual and dynamic (9, 16) and are influenced by diet, climate, age, and particularly weaning, which initiates a massive change in the intestinal microbiota (18, 19). Schierack et al. (21) showed that the swine gut acts as a reservoir for ExPEC and suggested that high numbers of ExPEC-typical VAGs correlate with intestinal colonization. This finding was possible only because of detailed analysis, which had not been considered previously for the analysis of intestinal microbiota of conventionally raised swine (9, 20, 21).

To gain further insight into the effects between *E. faecium* and *E. coli*, we investigated the *E. coli* population of young piglets in more detail by testing the influence of this probiotic at three different age periods and by sampling three different habitats of the gut: mucosa and digesta of the *colon ascendens* and feces. By quantifying defined *E. coli* clones and linking their phylogenetic background with possession of 69 VAGs, we aimed to identify possible shifts in the occurrence of certain clones between these three habitats. We hypothesized that *E. faecium* has an influence on *E. coli* colonization in the porcine intestine. While our data did not show changes in the overall diversity of *E. coli*, *E. faecium* feeding caused

Received 17 September 2013 Accepted 2 October 2013
Published ahead of print 11 October 2013
Address correspondence to Carmen Bednorz, carmen.bednorz@fu-berlin.de.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03138-13.

doi:10.1128/AEM.03138-13

a specific reduction in clones displaying ExPEC-typical virulence-associated factors. Since ExPEC-typical VAGs are known to promote colonization, this finding occurred particularly with clones adhering to the mucosa. Our results suggest a specific prophylactic effect of *E. faecium* against *E. coli*, with VAG profiles similar to those of ExPEC at the gut epithelia.

MATERIALS AND METHODS

Animal housing. Sixteen pregnant purebred landrace sows were divided into two groups: a control group ($n = 8$) and a probiotic group ($n = 8$). Sows of the probiotic group were fed a diet containing 4.2×10^6 to 4.3×10^6 CFU/g *E. faecium* NCIMB 10415 (Cylactin, Cerbios-Pharma SA, Lugano, Switzerland) from 28 days *ante partum* (a.p.) onwards as described previously (22). All animals were kept under similar conditions but in different stables to prevent the transmission of *E. faecium* via feces from the probiotic group to the control group. After birth, piglets were kept with their dams until weaning at the age of 26 ± 1 days. After weaning, when sows were separated from their litters, the piglets were kept in commercial flat deck pens in two different buildings with two animals per pen. This is a customary procedure in animal feeding trials to reduce cage effects. From the age of 12 days onward, piglets had access to a prestarter diet. Postweaning, the piglets were fed a starter diet. The starter diets of the probiotic supplemented group contained 5.1×10^6 CFU/g (prestarter) and 3.6×10^6 CFU/g (starter) of *E. faecium* NCIMB 10415. No antibiotics, either for therapeutic or prophylactic purposes, were applied to any of the animals used in the study.

Of all piglets from 16 sows (8 sows per feeding group), 24 piglets were randomly chosen and assigned to the probiotic and control groups. The influence of *E. faecium* was examined at three different ages (12, 26, and 34 days) and in two different samples from intestine and feces. Samples taken at the ages of 12 ± 1 ($n = 4$/feeding group), 26 ± 1 ($n = 4$/feeding group), and 34 ± 1 ($n = 4$/feeding group) days were used to obtain intestinal digesta and mucosal samples. Euthanasia and sampling were performed as described previously (22). In brief, following a midline abdominal incision, the small intestine was dissected from the large intestine at the ileocecal junction and both segments were dissected from the mesentery. Digesta and mucosal scrapings were taken from the *colon ascendens*. Fecal samples were obtained from the *ampulla recti* prior to euthanasia.

The study was approved by the local state office of occupational health and technical safety, Landesamt für Gesundheit und Soziales Berlin (LaGeSo no. 0347/09).

Isolation of *E. coli*. Isolation of *E. coli* has been described previously (23, 24). Briefly, intestinal contents from *colon ascendens* and feces were suspended in phosphate-buffered saline (PBS) buffer (0.2 g in 6 ml PBS), and serial dilutions were plated to different solid media to identify as many phenotypically diverse *E. coli* isolates as possible. To achieve this, sheep blood and Gassner agar plates (Sifin, Berlin, Germany), as well as CHROMagar orientation plates (CHROMagar, Paris, France[25]), were chosen. Additionally, CHROMagar orientation plates containing five different antibiotics (one per plate) were used: ampicillin (≥32 mg/ml), streptomycin (≥64 mg/ml), chloramphenicol (≥32 mg/ml), gentamicin (≥16 mg/ml), and tetracycline (≥16 mg/ml). The breakpoint concentrations were estimated based on previously published data (26). Thus, a total of eight different media were used to isolate *E. coli* bacteria from the three different samples from a total of 24 piglets. Colonies showing a typical pink color on CHROMagar orientation and/or a blue color on Gassner agar plates after incubation at 37°C for 24 h were assumed to be *E. coli* isolates. Approximately 20 pink or blue colonies per specimen (each representing a single isolate) were randomly picked from the plates (3 to 4 colonies per plate) for subcultivation onto CHROMagar orientation and sheep blood agar plates and incubated at 37°C for 24 h.

For mucosal samples, an approximately 2- by 5-cm section was washed twice in 1× PBS to remove visible fecal material. These short washing steps are not expected to affect mucosa-attached bacteria, since other studies have reported high numbers of mucosa-attached or epithelial cell-attached *E. coli* bacteria after up to four to six washing steps with physiological saline (27, 28). Approximately 0.5 g of each mucosal sample was removed from connective tissue by scraping with a glass microscope slide. Mucosal samples were transferred to a Dounce homogenizer and homogenized in 5 ml 1× PBS, and serial dilutions of the homogenates were plated to agar plates as described above.

A total of 20 to 24 *E. coli* isolates (each) were collected from the mucosa and intestinal content of the *colon ascendens* as well as the feces of each piglet.

Assignment of *E. coli* isolates to clones. Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) (23) using the restriction endonuclease XbaI was initially used to define clones to exclude analysis of duplicates. PFGE profiles were compared using BioNumerics software, version 6.6 (Applied Maths, Belgium), with the unweighted-pair group using average linkages method. Dice similarity indices (complete linkage; optimization, 1%; position tolerance, 1.5%) were also calculated.

Each *E. coli* colony was regarded as an individual isolate. A clone was defined as an *E. coli* group of isolates with a specific macrorestriction pattern, whereas two clones differed by more than one band (23). The diversity of the *E. coli* population was determined using Simpson's index of diversity (*Di*). This method enables comparison of the diversities of populations with different numbers of isolates and has been described previously (29, 30).

A major clone was defined as a clone which represented ≥50% of typed isolates in one sample, and a minor clone was defined as a clone which represented ≤10% of typed isolates in one sample (31). One representative isolate for each clone was randomly chosen for further analysis via multilocus sequence typing (MLST) and multiplex PCR.

MLST. MLST of one representative of each clone was performed to analyze the phylogenetic background. MLST was carried out as described previously (32). Gene amplification and sequencing were performed using primers available on the *E. coli* MLST website (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli). Sequences were analyzed using the software package SeqSphere 0.9.19 (http://www3.ridom.de/seqsphere). Sequence types (STs) were computed automatically. The phylogenetic group of the *E. coli* isolates was determined using the software program Structure 2.3.4 based on the sequences of the seven housekeeping genes used for MLST (http://pritch.bsd.uchicago.edu/structure.html). A spanning tree was constructed using BioNumerics (version 6.6; Applied Maths, Belgium).

Virulence-associated gene determination using PCR. The presence of a total of 69 VAGs was tested by multiplex PCR as previously described (33, 34). In addition, the presence of *stx*$_{2e}$, *faeG*, *fanA*, *fasA*, *fedA*, *fimF41a*, *est*-Ib, *est*-II, *elt*-Ia (typical for ETEC and edema disease *E. coli* [EDEC]), *aggR*, and the virulence plasmid pAA (typical for enteroaggregative *E. coli* [EAEC]) was assayed using primers and conditions described previously (35, 36). A representative isolate subjected to MLST was tested for VAGs, and results were considered to hold true for all isolates of the respective clone.

Statistical analysis. Statistical analyses were carried out using the software program SPSS 19.0 (IBM SPSS Statistics) and the R software environment, version 2.15.2 (http://www.r-project.org). The prevalence of 69 VAGs in the control and probiotic groups was determined and compared for both groups using a permutation test. In a second approach, we categorized all clones into three groups. The first group consisted of clones occurring in the control group only. The second group consisted of clones that were found only in samples from piglets of the probiotic group, and for a third group, only clones that occurred in piglets of both study groups (shared clones) were assigned (see Fig. 1). The prevalences of the genes considered for each VAG in these three groups were determined and also compared by a permutation test. We permuted the allocation of the piglets to the feeding groups 10,000 times and calculated chi-square statistics for each of the permuted samples. The proportion of the chi-square values that were greater than the chi-square statistic of the original sample was determined. A P value of <0.05 was considered significant. We also used the permutation test approach to compare the three habitats with respect

TABLE 1 Overview of distribution of all isolates, clones, and STs for the three different ages and habitats of the control and probiotic groups

Sample location	Age of piglets (days)[a]	No. of piglets[b]		Total no. of:					
				E. coli isolates[c]		Clones identified[d]		STs identified[e]	
		Control	Probiotic	Control	Probiotic	Control	Probiotic	Control	Probiotic
Colon (mucosa)	12 ± 1	4	4	93	83	26	30	21	24
	26 ± 1	4	4	88	83	35	31	26	22
	33 ± 1	4	4	78	81	28	24	21	18
Total (mucosa)		12	12	259	247	89	85	68	64
Colon (digesta)	12 ± 1	4	4	83	83	24	24	20	18
	26 ± 1	4	4	89	90	34	37	24	25
	33 ± 1	4	4	83	89	26	23	21	17
Total (digesta)		12	12	255	262	84	84	65	60
Feces	12 ± 1	4	4	43	42	16	14	12	11
	26 ± 1		4	86	88	37	35	29	25
	33 ± 1	4	4	65	89	25	28	18	23
Total (feces)		12	12	194	219	78	77	59	59
Total (control/probiotic)		12	12	708	728	251	246	192	183

[a] Mean ± SD.
[b] Samples were obtained from a total of 24 piglets.
[c] A total of 1,436 *E. coli* isolates were analyzed.
[d] Clones are defined as an *E. coli* group of isolates with a specific macrorestriction pattern, wherein two clones differed by more than one band. A total of 499 clones were found, with 168 different clones identified.
[e] STs, sequence types, based on seven housekeeping genes. A total of 375 STs were found, with 79 different STs identified.

to the occurrence of clonal STs. We used the permutation test approach since the analyses were carried out at the level of clones. Since clones belonging to the same piglet are not independent statistical units, we could not use the classical tests. Phylogenetic analysis was carried out using the software program STRUCTURE (37, 38). This software applies Bayesian methods to predict distinct groupings of the *E. coli* population. Minimum spanning trees were created by the program BioNumerics (version 6.6; Applied Maths, Belgium). The level of significance was $\alpha = 0.05$. Since the statistical analysis is exploratory, we did not perform a Bonferroni adjustment of the level of significance.

RESULTS

***E. coli* isolates from the probiotic group versus those from the control group. (i) Isolation of *E. coli*.** A total of 1,436 *E. coli* isolates were obtained from digesta, mucosa, and fecal samples from 24 clinically healthy piglets, 12 each from the control and the *E. faecium* (probiotic) groups. From each piglet, between 60 and 70 (20 to 24 per sample) *E. coli* isolates were analyzed, with approximately equal numbers of isolates from each feeding group (708 from the control versus 728 from the probiotic group) (see Fig. 1). The proportion of isolates originating with digesta was 36.1%; 35.1% were from mucosa, and 28.8% were from feces. As outlined in Table 1, the habitats of the isolates were also equally distributed between the feeding groups.

(ii) Clonal analysis of *E. coli*. Macrorestriction analysis of the 1,436 isolates identified 168 clones, with numbers of isolates per clone ranging between 1 and 181. Both feeding groups displayed a high clonal diversity, and their diversity indices (*Di*) were nearly equal (for *E. coli* populations from both feeding groups, $Di = 0.967$; for *E. coli* populations from the control group, $Di = 0.954$; for *E. coli* populations from the probiotic group, $Di = 0.962$). The diversity indices of the *E. coli* populations from the three different habitats also showed no major differences (for *E. coli* populations from digesta of the control group, $Di = 0.947$; for *E. coli* populations from mucosa of the control group, $Di = 0.946$; for *E. coli* populations from feces of the control group, $Di = 0.969$; for *E. coli* populations from digesta of the probiotic group, $Di = 0.961$; for *E. coli* populations from mucosa of the probiotic group, $Di = 0.965$; for *E. coli* populations from feces of the probiotic group, $Di = 0.958$). Of the 168 *E. coli* clones, 63 were isolated only from the control and 62 only from the probiotic group. Forty-three clones appeared in both groups, leading to a total of 86 clones in the control group and 82 clones in the probiotic group (see Fig. 1).

When each clone was counted once per group, a total of 106 clones were identified in the control and 105 in the probiotic group. In general, some clones occurred only in one animal and one sample, while other clones were present in up to 20 animals and 48 samples. On average, 2.4 clones per animal and 1.3 clones per sample were found. Two different major clones and 165 minor clones were detected, using the previous definition of minor and major clones (31). One representative isolate of each clone was randomly chosen for subsequent analyses, including multilocus sequence typing (MLST) and PCR for detection of virulence-associated genes (VAG-PCR).

(iii) Phylogenetic analysis. Macrorestriction analysis has a higher discriminatory power than MLST, conferring the ability to subdivide sequence types (STs) into clones. With the stringent definition of the clone, on which this study is based, we performed MLST (32) analysis to assign an ST to each of the 168 clones. This resulted in the identification of 79 distinct STs. Eighteen of these showed allele combinations which had not been reported as of 2 March 2013 on the MLST website (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli). For relatedness of all STs, see Fig. 2A. The minimum spanning tree (MSTree) comprises all 1,436 isolates, belonging to the 168 clones, which are assigned to 79 STs. Forty-four different STs occurred in the probiotic group and 50 different STs in the control group, with 30 STs occurring in both feeding groups. STs

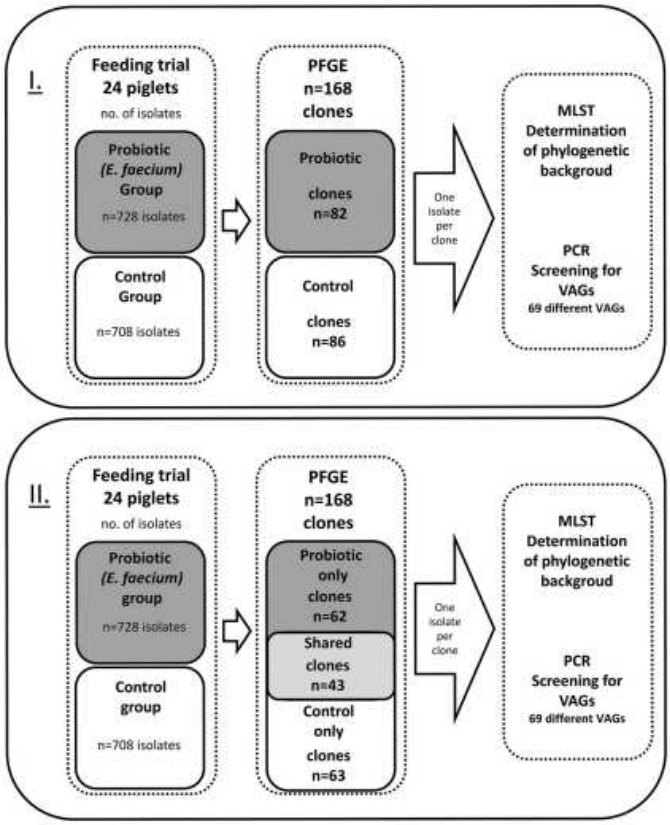

FIG 1 Schematic work flow of clones and isolates included and methods used in this work. Two different approaches were used: I, comparing clones from the two feeding groups, the probiotic and control groups; II, comparing clones present in either the control or probiotic group or shared by both groups.

were nonrandomly distributed, since some STs were overrepresented, e.g., ST10 (n = 18 clones; n = 125 isolates), ST58 (n = 10 clones; n = 118 isolates), ST167 (n = 3 clones; n = 114 isolates), and ST2496 (n = 4 clones; n = 233 isolates). The number of isolates per ST varied from a maximum of 233 (ST2496) to a minimum of just 1. These singletons were found in a total of 17 STs; however, an association with a particular feeding groups was not defined.

Evidence suggests that specific *E. coli* phylotypes are associated with certain ecological adaptations (39). To define phylotypes, we analyzed the recombination events of *E. coli* by comparing the polymorphisms in the concatenated sequences of the seven gene fragments used for MLST using the STRUCTURE software program (37, 38). This software applies Bayesian methods to predict distinct groupings of the *E. coli* population. Using the linkage model of STRUCTURE, we were able to assign the isolates to ECOR groups A (59 clones, 710 isolates), B1 (27 clones, 197 isolates), B2 (4 clones, 12 isolates), and D (8 clones, 116 isolates). In addition, 31 clones (203 isolates) were assigned to the hybrid group AxB1 and 39 clones (198 isolates) to hybrid group ABD. Thus, we defined a remarkably low number of isolates belonging to ECOR B2 and D. Both phylogroups are known to harbor particularly virulent *E. coli* strains (15, 32, 40, 41). Most importantly, no differences were found between the two feeding groups, since all phylogenetic groups were distributed equally between the probiotic group and the control group (for data, see Fig. 2A and B).

We further analyzed the distribution of STs in association with the habitat the *E. coli* bacteria were isolated from, namely, mucosa, digesta, or feces. However, no differences were seen in the distribution (see Fig. S1 in the supplemental material). Also, we found no age-related association of the piglets with the appearance of certain STs (data not shown).

(iv) VAG determination. As outlined in Fig. 1, one representative isolate of each of the 168 *E. coli* clones was further tested by PCR for the presence of 69 virulence-associated genes (VAGs) to assign InPEC and ExPEC pathotypes. Using PCR-based detection of *est*-Ia, *est*-II, *eltB*, *fedA*, *fasA*, stx_1, stx_2, or *eae* and *bfp*, none of the isolates could be assigned to the highly pathogenic edema disease *E. coli* (EDEC) or the enteropathogenic *E. coli* (EPEC), while 1.2% of the clones and 0.2% of the isolates were identified as Shiga

TABLE 2 VAGs that are differently distributed between control and probiotic groups[a]

Origin	Gene (function/location)	% positive samples for group		*P* value[b]
		Probiotic	Control	
All	*hlyF* (toxin)	14.1	23.3	0.011
	focG (adhesin)	1.9	6.8	0.015
	papC (adhesin)	0.4	4.1	0.008
	papGIII (adhesin)	0.3	2.8	0.028
	iroN (iron acquisition/plasmid)	23.4	33.3	0.040
	cvaC (bacteriocin/plasmid)	12.9	23.9	0.002
Mucosa	*hlyF* (toxin)	15.0	23.6	0.046
	focG (adhesin)	0.4	7.0	0.002
	papC (adhesin)	0.0	4.3	0.009
	sitA (iron acquisition)	42.7	57.4	0.045
	cvaC (bacteriocin/plasmid)	13.8	23.6	0.041
Digesta	*hlyF* (toxin)	14.1	25.5	0.012
	focG (adhesin)	2.7	8.6	0.037
	papC (adhesin)	0.4	3.5	0.027
	papGIII (adhesin)	0.4	2.7	0.023
	iroN (iron acquisition/plasmid)	22.9	34.5	0.011
	cvaC (bacteriocin/plasmid)	12.6	25.9	0.001

[a] Calculations based on all isolates of each group for VAGs that are differently distributed between mucosa and digesta from control and probiotic groups.
[b] A total of 69 VAGs were screened; here, only significant results are listed (permutation test).

toxin-producing *E. coli* (STEC) (detection of stx_1 and stx_2), 6.5% clones and 1.5% isolates as atypical EPEC (aEPEC) (*eae*-positive and *bfp*-negative), and 4.8% clones and 1.5% isolates as enterotoxigenic *E. coli* (ETEC) (detection of *est*-II, *est*-Ia, *eltB*, *fedA*, or *fasA*). Isolates with VAGs described as associated with ExPEC pathotypes were more frequently detected. However, compared to findings of previous studies, the number of intestinal pathogenic *E. coli* identified in piglets in this study was low (20, 21).

In general, we detected only a limited number of VAGs in all isolates from the feeding groups, with a tendency toward lower numbers in isolates from the probiotic group. However, a more detailed analysis revealed differences in the appearance of six VAGs between all isolates of the probiotic group on the one hand and all isolates from the control group on the other hand: *hlyF* ($P = 0.011$), *focG* ($P = 0.015$), *papC* ($P = 0.008$), *papGIII* ($P = 0.028$), *iroN* ($P = 0.04$), and *cvaC* ($P = 0.002$) were significantly less frequent in the probiotic group (Table 2). These genes represent four different categories of VAGs (toxin, adhesion, iron acquisition, and bacteriocin), all of which are typical for ExPEC (33, 34). Some of these genes, like *iroN*, *tsh*, or *colV*, do not appear independently from each other since they are located on plasmids (15). Dividing the isolates of the two feeding groups according to the habitat they had been isolated from, namely, mucosa, digesta, and feces, a more distinct picture arises. Concentrating on the isolates from fecal samples, no significant differences appeared. In contrast, *E. coli* isolated from digesta showed significantly different distributions of the six VAGs mentioned above. The isolates from mucosa showed nearly equal significant associations, with only one exception: *papGIII* ($P = 0.138$) was not significantly different in occurrence (not shown), but another gene, *sitA* ($P = 0.045$), was reduced in these specific isolates (Table 2).

Further analyses were performed to detect possible differences between isolates in association with the age of the piglets (before, at the time of, and after weaning). Here, the total number of *E. coli* isolates was higher in weaned piglets, and they harbored more toxin and adhesin genes, with the toxin genes *astA* ($P = 0.005$) and *est*-II ($P = 0.028$) and adhesion genes *mat* ($P = 0.040$) and *traT* ($P = 0.000$) being significantly increased after weaning (see Table S1 in the supplemental material).

***E. coli* isolates unique to the probiotic group versus those unique to the control group versus the shared group.** In summary, we detected only minor differences when comparatively analyzing those *E. coli* isolated from the control group versus those isolated from the probiotic feeding group. To gain further insight into possible group specificities of the identified *E. coli* clones, we divided the 168 clones into three groups depending on their occurrence: those restricted to piglets from the probiotic (probiotic only; $n = 62$) or the control group (control only; $n = 63$) and those shared by both groups (shared; $n = 43$) (Fig. 1). The distribution of STs was as follows: $n = 20$, probiotic only; $n = 29$, control only; $n = 30$, shared group. Thus, despite this new assignment, the clones and STs were equally distributed between the three groups (Fig. 2).

Virulence-associated gene determination. Based on assigning the clones into these three groups (probiotic only, control only, and shared), the occurrences of the 69 VAGs revealed only marginal differences. As outlined in Table S2 in the supplemental material, only seven of these VAGs, mainly genes coding for adhesins, showed significant differences. However, when looking at the distribution of the *E. coli* VAGs in association with the ecological-habitat origin of the samples, namely, mucosa, digesta, or feces, clear differences were found. A total of 11 VAGs (*tsh* [$P = 0.017$], *mat* [$P = 0.001$], *focG* [$P = 0.002$], *papC* [$P = 0.037$], *colV* ′[$P = 0.048$], *ompT* [$P = 0.003$], *cvaC* [$P = 0.004$], *iroN* [$P = 0.000$], *etsB* [$P = 0.003$], *etsC* [$P = 0.003$], and *hlyF* [$P = 0.001$]) were significantly reduced in clones adhering to the mucosa from the probiotic or shared groups compared to results for the control group (Table 3). As mentioned above, some of the named genes are located on plasmids and are nonrandomly distributed. Nevertheless, ExPEC-typical VAGs for adhesins, serum resistance, and iron acquisition appeared to be affected, indicating a reduction in the frequency of *E. coli* bacteria similar to ExPEC in the *E. faecium*-supplemented group of piglets.

DISCUSSION

The probiotic *E. faecium* NCIMB 10415 is widely used as a feed supplement for different animals and as a pharmaceutical in humans. Previous studies have reported favorable effects on both health and shedding of pathogenic microorganisms (8, 10, 11, 42–45). We had previously observed a reduction of *E. coli* serotype O141 during supplementation of *E. faecium* NCIMB 10415 in piglets (10). However, serotyping is not sufficient for defining the pathogenic potential or the phylogenetic background of the respective *E. coli* bacteria. We therefore investigated the influence of *E. faecium* supplementation on the porcine intestinal *E. coli* populations by both clonal and phylogenetic analysis of 1,436 *E. coli* isolates and PCR-based typing of virulence-associated genes (VAGs) typical for intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC). Our analysis focused on three main approaches, namely, comparison of the following *E. coli* populations: (i) those originating with the control group ver-

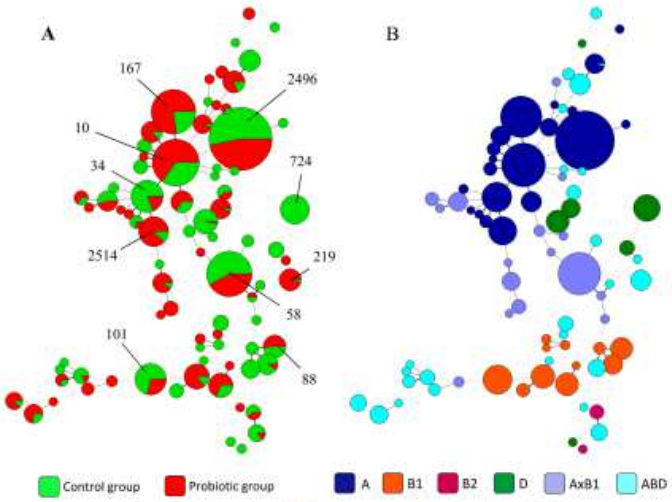

FIG 2 (A) Minimum spanning tree (MSTree) of STs from 168 clones defined by PFGE, assuming the results for one representative for the complete group. Green, control; red, probiotic group. (B) Minimum spanning tree of phylogenetic groups, assuming the results for one representative for the complete group. Both MSTrees were calculated using Bionumerics 6.6.

sus those from the probiotic group, (ii) those originating with feces versus digesta versus mucosa, and (iii) those present either in the control or the probiotic group or shared by both groups.

Only by this extensive clonal analysis of each of the 1,436 isolates were we able to show that *E. faecium* feeding is associated with reduced appearance of pathogenic *E. coli* bacteria, in particular those with features of ExPEC, on the mucosa and the digesta of the *colon ascendens*.

Macrorestriction analysis revealed an overall high clonal diversity of *E. coli* in the 24 tested piglets, confirming previous observations (9, 16, 46). Nevertheless, our results indicated that the supplementation of *E. faecium* NCIMB 10415 to healthy piglets did not influence the overall intestinal *E. coli* diversity, corroborating previous data (9, 10). Likewise, a detailed breakdown of the *E. coli* clones into three different groups (control only, probiotic only, and shared), did not indicate any significant changes in diversity.

Determination of 69 VAGs revealed an overall low number of VAGs regardless of the feeding regimen. The low occurrence of pathogenic *E. coli* harboring VAGs correlates with a low number of isolates belonging to ECOR B2 and D, two phylotypes which are known to harbor virulent *E. coli* types (15, 32, 41, 47). We consider the low number of VAGs identified to be at least partly due to the optimized hygienic conditions under which the animals were

TABLE 3 Association between mucosa-associated *E. coli* isolates from the probiotic, control, or shared group and the occurrence of VAGs[a]

	Mucosa-associated *E. coli* isolated from:									
	Probiotic-only group[b]			Control-only group[c]			Shared group[d]			
Gene(s) (function/location)	No. neg.	No. pos.	% pos.	No. neg.	No. pos.	% pos.	No. neg.	No. pos.	% pos.	*P* value[e]
tsh (adhesin/plasmid)	84	21	20.0	104	38	26.8	253	4	1.6	0.017
mat (adhesin)	22	83	79.0	6	136	95.8	102	155	60.3	0.001
focG (adhesin)	104	1	1.0	124	18	12.7	257	0	0.0	0.002
papC (adhesin)	105	0	0.0	131	11	7.7	257	0	0.0	0.037
ColV genes: *cvi*, *cva* (plasmid)	89	16	15.2	103	39	27.5	233	24	9.3	0.048
ompT (outer membrane protein)	80	25	23.8	91	51	35.9	233	24	9.3	0.003
cvaC (bacteriocin/plasmid)	92	13	12.4	90	52	36.6	227	30	11.7	0.004
iroN (iron acquisition/plasmid)	73	32	30.5	59	83	58.5	220	37	14.4	0.000
etsB (ABC transporter)	72	33	31.4	89	53	37.3	226	31	12.1	0.003
etsC (ABC transporter)	72	33	31.4	89	53	37.3	226	31	12.1	0.003
hlyF (toxin)	87	18	17.1	86	56	39.4	233	24	9.3	0.001

[a] neg., negative; pos., positive.
[b] Probiotic only; $n = 105$.
[c] Control only; $n = 142$.
[d] Shared group; $n = 257$.
[e] A total of 69 VAGs were screened; here, only significant results are listed (permutation test).

housed. Genes associated with InPEC were also very rarely detected. Nevertheless, ExPEC-typical genes were present in the *E. coli* isolates, and differences were evident in our three main approaches. Concerning the two feeding groups, significantly lower numbers of six different VAGs (Table 2) were identified in the probiotic feeding group. Focusing on the habitats (feces versus digesta versus mucosa), significant changes were detected for the genes mentioned above in the digesta. Isolates originating from the mucosa differed by one gene. Most of the clones with ExPEC-typical VAGs appeared in the mucosa as well as the digesta. We therefore speculate that there is a dynamic exchange between the adherent and nonadherent *E. coli* in the *colon ascendens*. This could be influenced by *E. faecium* NCIMB 10415.

Applying a third analytical approach of dividing the clones into the three groups (probiotic only, control only, and shared), we found the same and even more significant reductions only of VAGs for mucosa-associated isolates. The occurrence of 11 genes was reduced in both the probiotic and shared groups; in particular, genes for adhesion, serum resistance, and iron acquisition were significantly decreased (Table 3). This finding was perhaps not unexpected, since *E. coli* bacteria adhering to the mucosa should have adhesive properties. The reduction in genes associated with extraintestinal virulence suggests that feeding of *E. faecium* can reduce the number of *E. coli* bacteria harboring ExPEC-typical genes in the intestine, especially those adhering to the mucosa of the *colon ascendens*.

Although some of the genes examined are linked to each other via their plasmid-borne nature, most of these genes are not perfectly linked, resulting in different *P* values from those which are linked due to their colocalization on plasmids.

Several hypotheses regarding how the probiotic *E. faecium* NCIMB 10415 may influence the bacterial colonization of piglets have been suggested. One hypothesis is based on the assumption that enterococci can inhibit strains in the gut by production of organic acids and by lowering the pH (44). This explanation would appear to be unlikely, since we did not observe an overall reduction of ExPEC features; rather, the decrease of genes associated with extraintestinal virulence was restricted to the mucosa and digesta only. Were this explanation correct, we should have observed a reduction of *E. coli* with ExPEC-typical VAGs in the feces as well. One might hypothesize that a pH effect of the probiotic has a greater influence in the *colon ascendens*, an effect which decreases during transit through the intestine. Due to the short generation time of *E. coli*, the ExPEC-typical subpopulation in the feces may already have recovered, masking an effect of the probiotic strain on ExPEC in the feces.

Another explanation for the probiotic effect is the inhibition of attachment by the pathogen to the mucosa of the gastrointestinal tract (48). This has been described for members of the order *Lactobacillales*, including the lactobacilli (49, 50) and enterococci (51, 52). According to this hypothesis, probiotic bacteria compete with pathogens for binding sites on the host cell surfaces or bind to the pathogen itself, blocking adhesive surface structures or preventing binding of the pathogen to binding sites by sterical interference (51, 53). The competition leads to a reduction in the adhesive properties of the pathogen and thus to its decreased virulence. This assumption could explain our results, since a higher adhesion rate of *E. faecium* than of *E. coli* similar to ExPEC to gut epithelium or binding of *E. faecium* to *E. coli* would lower the number of *E. coli* bacteria with ExPEC-typical genes adhering to the mucosa. In a previous study, gnotobiotic pigs were infected with pathogenic *E. coli*. Pigs supplemented with *E. faecium* NCIMB 10415 showed a reduction in diarrhea caused by *E. coli* and a greater increase in body mass (43).

The finding that ExPEC-typical genes were reduced in piglets supplemented with *E. faecium* was possible only due to the fact that we sampled the mucosa and digesta of the piglets and did not limit the sampling to feces. Nevertheless, most studies focus on the collection and analysis of fecal samples (17, 30, 46, 54) or the intestinal content (46, 55). The practice of examining feces is common because it presents a rapid and easy method for conducting such studies, avoiding euthanasia of the tested animals. However, our data question the relevance of fecal sampling only, since our results clearly show that fecal samples do not appear to include the full population of *E. coli*. Since important subpopulations might not be sampled with this method, changes in their genotypic or phenotypic appearance would not be detected.

Weaning presents a stress factor for piglets and is known to influence the intestinal microbiota (8, 12, 17, 30, 56). Therefore, we expected higher numbers of pathogenic *E. coli* bacteria in the intestine of the weaned animals. A possible explanation for the observation of low pathogen numbers in the animals in this study might be effective stall management, resulting in improved health status. Beginning with weaning until the end of the study, the piglets were kept in pairs per pen. This situation is not comparable to the typical livestock breeding conditions. Whether the effect of the probiotic would be stronger or weaker when supplemented under typical farming conditions should be tested in further studies. Since *E. coli* harboring higher numbers of ExPEC-typical VAGs might have been expected, one might hypothesize that an effect on the mucosa-associated isolates would be stronger.

In conclusion, *E. faecium* feed supplementation caused no evident changes in the overall diversity of *E. coli* in healthy weaned piglets, corroborating previous data (9, 22, 57–59). However, more detailed analyses showed a reduction in isolates harboring ExPEC-typical virulence-associated factors, particularly for isolates adherent to the mucosa of the *colon ascendens*. Since ExPEC bacteria are known to have a significant colonization advantage (21), our results suggest a prophylactic effect of *E. faecium* NCIMB 10415 against potential pathogenic *E. coli* at the intestinal epithelial mucosa.

ACKNOWLEDGMENTS

The study was supported by the German Research Foundation (DFG) through grant SFB852/1. Torsten Semmler and Bianca Kinnemann were supported by the Federal Ministry of Education (BMBF) and Research Network Zoonosis (FBI-Zoo, grant 01KI1012A).

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2.2.2. Contribution

I designed, structured, and prepared the manuscript and the experiments described in the publication. Additionally, I discussed and interpreted the results. The final manuscript version was prepared by me after having discussed it with all the authors mentioned on the manuscript. SG took part in writing parts of the manuscript, drafted and critically revised the manuscript for important intellectual content and gave final approval of the version to be published. In addition, he took part in adjusting the text. KO helped with preparing for the animal trials, plating the samples, and picking the colonies. Further, she helped me with the experiments, such as PFGE, VAG – PCR and MLST. BK helped with the experiments, such as PFGE and MLST. RP was responsible for the concept of the animal trials and he drafted and critically revised the manuscript. SH drafted and critically revised the manuscript. KT drafted and critically revised the manuscript. TS helped with the analysis of sequence data of the MLST and with the figures. KN did the statistical analysis. PS was responsible for the concept of the project and drafted and critically revised the manuscript. AB drafted and critically revised the manuscript. LHW drafted and critically revised the manuscript for important intellectual content and gave final approval of the version to be published. In addition, he took part in writing parts of the manuscript, in structural arrangement of the paragraphs and he was responsible for the concept of the project.

All authors read and approved the final manuscript.

2.2.3. Summary of Publication 2

Feed supplementation with the probiotic *Enterococcus faecium* for piglets has been found to reduce pathogenic gut microorganisms. Since *Escherichia coli* is among the most important pathogens in pig production, we performed comprehensive analyses to gain further insight into the influence of *E. faecium* NCIMB 10415 on porcine intestinal *E. coli*. A total of 1,436 *E. coli* strains were isolated from three intestinal habitats (mucosa, digesta, and feces) of probiotic-supplemented and non-supplemented (control) piglets. *E. coli* bacteria were characterized via pulsed-field gel electrophoresis (PFGE) for clonal analysis. The high diversity of *E. coli* was reflected by 168 clones. Multilocus sequence typing (MLST) was used to determine the phylogenetic background, revealing 79 sequence types (STs). Pathotypes of *E. coli* were further defined using multiplex PCR for virulence-associated genes. While these analyses discerned only a few significant differences in the *E. coli* population between the feeding groups, analyses distinguishing clones that were uniquely isolated in either the probiotic group only, the control group only, or in both groups (shared group) revealed clear effects at the habitat level. Interestingly, extraintestinal pathogenic *E. coli* (ExPEC)-typical clones adhering to the mucosa were significantly reduced in the probiotic group. Our data show a minor influence of *E. faecium* on the overall population of *E. coli* in healthy piglets. In contrast, this probiotic has a profound effect on mucosa-adherent *E. coli*. This finding further substantiates a specific effect of *E. faecium* strain NCIMB 10415 in piglets against pathogenic *E. coli* in the intestine. In addition, these data question the relevance of data based on only sampling fecal *E. coli* as a means to judge intestinal *E. coli* microbiota on the whole.

3. Unpublished data

3.1. Statistical analysis of *E. coli* colony counts from the zinc feeding trial (Study 1)

The analysis was performed to verify whether zinc feeding influences the overall *E. coli* number in the intestine of pigs. Vahjen et al. (2011) already showed a diverse response of the bacterial core to ZnO supplementation. Lactobacilli were reduced whereas species of the Enterobacteriaceae were increased. An influence of zinc on the *E. coli* number may be an explanation for further results, such as an enhanced plasmid uptake. A higher density of *E. coli* could boost HGT *(Stecher et al., 2012).*

All samples taken from the pigs in the zinc trial were stricken out on agar plates (see Publication 2). The coliform colonies were counted on all plates and statistically analyzed with SPSS 20.0 (IBM SPSS Statistics). For this analysis the Mann Whitney U test was used for all feeding groups in this trial (control group [50 ppm], the low zinc group [150 ppm], and the high zinc group [2,500 ppm]). The results gained through isolation from CHROMagar orientation plates (Chromagar, Paris, France) are shown in Fig. 4. The results for coliform colonies from the piglets were divided into the feeding groups (Fig. 4A), samples from the intestine (Fig. 4B), and the three different age groups of the piglets (Fig. 4C). This was done to check for zinc concentration dependency, distribution in the small and large intestine and age-dependent effects of *E. coli* numbers.

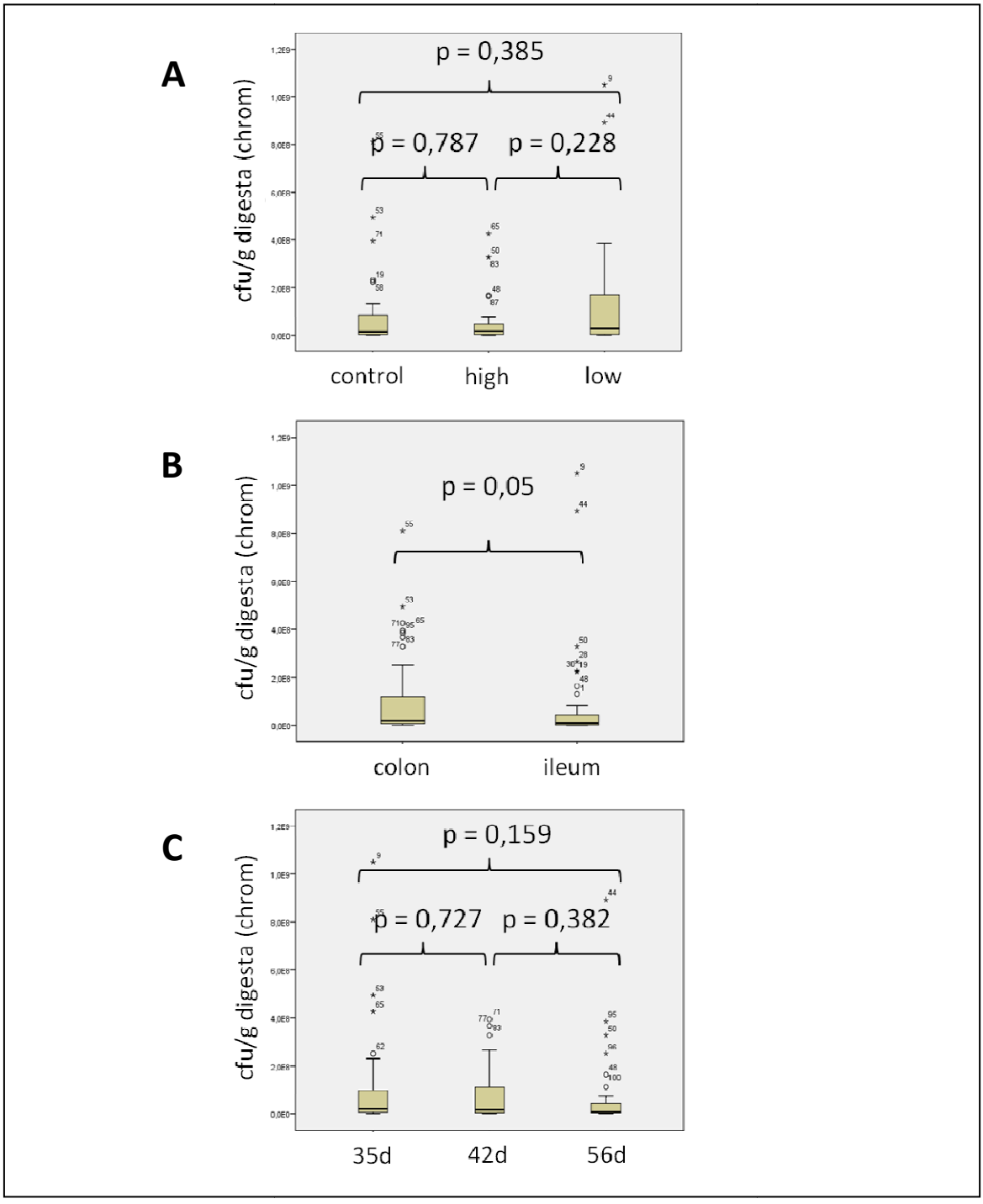

Fig. 4: Statistical analysis of *E. coli* numbers in the intestine of piglets with different zinc concentrations, part of intestine, and age. The colony forming units (cfu) per gram (g) of *E. coli* isolated from the digesta (Ileum and Colon as.) were determined for each piglet. *E. coli* numbers were counted on CHROMagar™ plates (chrom) with three different dilutions (see Tab. 1/ 6 plates in total for each sample) from Ileum (ileum) and Colon as. (colon). P-values were determined by the Mann Whitney U test with SPSS 20.0. Figure 4A shows the cfu/g and the p-values for the three feeding groups: control group (control), high zinc group (high), and low zinc group (low). Figure

4B shows the cfu/g and the p-value for the two different samples (ileum and colon) taken from each piglet. Figure 4C shows cfu/g and the p-values for the three age groups of the piglets: 35 day-old (35d), 42 day-old (42d), and 56 day-old (d).

Quantification of the *E. coli* population revealed that the total numbers of this bacterium did not significantly differ between the feeding groups investigated ($p > 0.05$). Thus, the total amount of *E. coli* per gram digesta did not vary according to the zinc supplementation, regardless of the zinc concentration (Fig. 4A). However, as expected, *E. coli* numbers were significantly higher in the ascending colon compared to the ileum ($p < 0.05$; Fig. 4B). Several studies demonstrated that *E. coli* colonizes the small and large intestine. However, they prefer to colonize the large intestine of mammals due to the higher pH. In addition, the age of the pigs has no significant influence on *E. coli* numbers ($p > 0.5$; Fig. 4C). This important finding suggests that the observed changes in the diversity of the *E. coli* population were not influenced by the overall number of *E. coli*.

4. Discussion

The aim of this project was to investigate the impact of two different feed supplements on the intestinal *E. coli* population in pigs. The probiotic *E. faecium* NCIMB 10415 strain and the divalent metal ion zinc were used as feed supplements, each in independent animal trials. As a part of the CRC 852, effects of the feed supplements were studied in pigs as a most relevant livestock population. Pigs were used due to their importance in animal farming as they supply the main part of meat consumed in Germany. Furthermore, Germany is a main global provider of slaughtered pigs with ~ 58.4 million pigs in 2012 (Federal Statistical Office). Pigs are also a useful study subject for human diseases as has been shown in diabetes studies in gnotobiotic pigs *(Yang et al., 2010)*, as well as in other studies *(Fairbairn et al., 2011)*. Pigs are omnivores like humans and their gastrointestinal tract is similar to that of humans *(Canavan et al., 1997)*. Thus, studying the pig's intestinal microbiome is of particular importance not only for the pig population itself. It also provides valuable knowledge for understanding the mechanisms of the human gut microbiome.

Several studies have already demonstrated effects of feed supplements on the microbiota and the immune system of pigs *(Mafamane et al., 2011; Pollmann et al., 2005; Scharek et al., 2005; Szabo et al., 2009)*. However, due to the high number of different disciplines involved in the CRC 852, it was possible to apply a systems biology approach with a rather high complexity. So far no previous research was as extensive as these studies of the CRC 852. All studies used mostly the same animals, were performed at the same time, and thus provide better comparable and stronger results than those gained from the other studies *(Twardziok et al., 2014)*.

4.1. Effects of used feed supplements on the *E. coli* population and the general microbiota of pigs

The two comprehensive studies described in Publications 1 and 2 showed diverse effects of the two feed supplements *E. faecium* and zinc on the *E. coli* population in pigs. *E. faecium* reduced the number of *E. coli* pathotypes on the mucosa (Publication 2), while zinc caused

a higher proportion of multi-resistant *E. coli* (Publication 1). This fact raises the question which supplement is more advisable based on the impact on *E. coli* and the microbiota. In relation to this, the possibility of negative side effects in the case of zinc should be kept in mind.

4.1.1. Probiotic *E. faecium* NCIMB 10415

The probiotic strain *E. faecium* NCIMB 10415 has already been part of several studies in pigs, displaying different effects on the microbiota *(Kreuzer et al., 2012b; Pollmann et al., 2005; Schierack et al., 2007; Szabo et al., 2009; Twardziok et al., 2014).*

4.1.1.1. Effects on *E. coli*

In previous studies effects on pathogenic *E. coli* in pigs have also been investigated. So far, most of them have reported probiotic effects on intestinal pathogenic *E. coli (Jin et al., 2000; Scharek et al., 2005; Taras et al., 2006; Underdahl et al., 1982).* These researchers described a reduced occurrence of diarrhea, pathogenic *E. coli* F4, or frequency of beta-hemolytic and O141 serovars of *E. coli* in the intestinal contents of probiotic piglets *(Jin et al., 2000; Scharek et al., 2005; Taras et al., 2006; Underdahl et al., 1982).* Additionally, broilers challenged with F4-positive *E. coli* (*E. coli* F4ac and F4MB) showed lower concentrations of *E. coli* when treated with *E. faecium* (HJEF005/NCIMB 10415) *(Cao et al., 2013; Samli et al., 2010).* The results from Publication 2 demonstrate that probiotics like *E. faecium* NCIMB 10415 can also reduce numbers of extraintestinal pathogenic *E. coli* (ExPEC), especially in samples from the mucosa of pigs. The mucus layers are thought to be the niche or natural habitat of *E. coli* in the intestine *(Abraham et al., 2012; Dixit et al., 2004; Schierack et al., 2008).* ExPEC are carried asymptomatically in the gut, but they can turn into pathogens when they exit the intestine, for example when they enter the blood circulation, as SEPEC, or the urinary tract, as UPEC *(Ewers et al., 2007).* In this case, *E. faecium* putatively reduce the risk of extraintestinal *E. coli* diseases by suppressing ExPEC in the intestine. Thus, in principle the probiotic bacterium *E. faecium* could have positive effects, reducing InPEC and ExPEC in the intestine of pigs.

In addition, the finding that the samples from mucosa had the highest reduction of typical VAGs for ExPEC, and the samples from feces no reduction, was unexpected. This is an important fact especially for the interpretation of other studies performed with the same

probiotic bacterium *(Bybee et al., 2011; Lappin et al., 2009; Marcinakova et al., 2006).* Because of the sampling, the question arises how significant and representative analyses that only use fecal samples are. Our study clearly demonstrated that fecal samples alone do not represent the conditions of the intestinal microbiota. There are many publications about effects of probiotics that are based on results from fecal samples only *(Bybee et al., 2011; Lappin et al., 2009; Marcinakova et al., 2006; Strompfova et al., 2006a).* As already discussed in Publication 2, it is less time consuming and not as expensive as taking more samples directly from the intestine, such as the mucosa or the digesta. However, to obtain precise and reliable results, it seems necessary to at least take samples from the intestine. This is thus another very important finding that further underlines the relevance of this work and should receive attention through the planning of new studies on the microbiota in animals. It also questions the meaningfulness of studies of the human microbiome, which are indeed usually based on fecal samples only *(Dicksved et al., 2014; Gomez-Moreno et al., 2014; van Zanten et al., 2014).*

4.1.1.2. Effects on microbiota and the host immune system

The studies performed within the CRC 852 analyzed various aspects of the microbiota and the immune system of the same pigs under investigation. While most studies were able to show clear effects *(Klingspor et al., 2013; Kreuzer et al., 2012b; Siepert et al., 2014; Starke et al., 2013a; Twardziok et al., 2014; Wang et al., 2014),* other investigators were not able to find such data *(Kreuzer et al., 2012a; Martin et al., 2012).*

One of the most interesting findings was the result from a systems biology analysis which gave strong evidence that effects of *E. faecium* supplementation most prominently affected the interplay between the intestinal microbiota and the intestinal immune system, in particular innate immunity *(Twardziok et al., 2014).* These effects clustered around weaning *(Twardziok et al., 2014),* which is the time when piglets are most susceptible to diarrhea *(Fairbrother et al., 2005).* This analysis was based on different results and parameters detected within the CRC 852 and gives a good summary of the main results. Pathogenic *E. coli* numbers and virus shedding, especially of Rotavirus, were decreased *(Bednorz et al., 2013a; Kreuzer et al., 2012b),* while *Lactobacillus (L.) amylovorus* and *L. acidophilus* numbers were increased under *E. faecium* supplementation *(Starke et al., 2013a).* Previous studies from the "DFG Forschergruppe 483" also found specific effects

of *E. faecium* on the microbiota, such as reduction of *Chlamydia* and *E. coli (Pollmann et al., 2005; Scharek et al., 2005; Taras et al., 2006).* Scharek et al. (2005) concluded that the probiotic *E. faecium* NCIMB 10415 influenced the early bacterial colonization of piglets and that this was reflected by a reduction of occurrence of enteropathogenic bacteria. Similar results could be detected in broiler chickens fed by probiotic *E. faecium* (HJEF005). Cao et al. (2013) showed in broilers challenged with F4-positive *E. coli* (*E. coli* F4ac and F4MB) that concentrations of *E. coli* and *Clostridium perfringens* decreased, whereas *Lactobacillus* counts and *Bifidobacterium* increased in the cecal contents of the probiotic group. They suggested that *E. faecium* can promote growth performance, improve intestinal morphology, and beneficially manipulate the cecal microbiota in broilers *(Cao et al., 2013).* Similar results were presented by Samli et al. (2010). They reported a significant decrease in the population of *E. coli* through the addition of *E. faecium* NCIMB 10415 in the ileal and caecal microbiota *(Samli et al., 2010).*

The CRC 852 showed that *E. faecium* affected the immune system of the host in different ways. *E. faecium* was able to beneficially influence humoral immune responses in pigs after vaccination and recovery from swine influenza virus (SIV) infection *(Wang et al., 2014).* Furthermore, *E. faecium* increased the absorptive and secretory capacity of jejunal mucosa and enhanced the intestinal barrier *(Klingspor et al., 2013).* Supplementation with *E. faecium* NCIMB 10415 affected intestinal immune-associated gene expression in challenge trials with *S.* Typhimurium, which is forced post-weaning when the animals receive increased levels of the probiotic. The reduction in gene expression may delay the host response to infections and offer pathogenic bacteria such as *Salmonella* an opportunity, leading to increased bacterial loads and shedding *(Siepert et al., 2014).* Kreuzer et al. (2012) could detect similar results in the case of *S.* Typhimurium infection. Furthermore, treatment with *E. faecium* could lead to a higher number of the pathogen in tonsils of infected animals *(Kreuzer et al., 2012a).* So far these results point towards a reduced inflammatory response caused by reducing T-cell activity, which would explain the higher *Salmonella* load as well as the higher antibody response against *Salmonella*.
Previous challenge studies performed by the “DFG Forschergruppe 483” also observed an enhanced course of infection in weaning piglets treated with *E. faecium*. The fecal excretion and organ colonization was higher in the probiotic treated group, indicating that the probiotic favored or tolerated the colonization of the *S.* Typhimurium strain. This fact

led to an increase in the adaptive immune response toward *Salmonella*, as the probiotic treatment appeared to result in greater production of specific antibodies (higher IgM and IgA levels). It is uncertain whether this increased humoral immune response was a result of feeding the probiotic or a result of elevated *Salmonella* loads *(Szabo et al., 2009)*. In another study with *E. faecium* a reduction of specific intracellular T-cells, reduced circulating T-cells, and a less efficient control of intracellular *Salmonella* growth mediated by peripheral blood mononuclear cells, were observed, which contributes to a more severe infection with *S.* Typhimurium *(Mafamane et al., 2011)*.

4.1.1.3. Possible mechanism of probiotic *E. faecium*

The probiotic *E. faecium* NCIMB 10415 may have different mechanisms that influence the microbiota and the immune system of piglets. Some hypotheses for the effects on pathogenic *E. coli* have already been suggested (Publication 2). The hypotheses are based on the assumption of an inhibition or reduction of adherence to the host cells. One hypothesis is based on the assumption that enterococci can inhibit strains in the gut by production of organic acids and by lowering the pH *(Strompfova et al., 2006b)*. Another explanation for the probiotic effect is the inhibition of attachment of the pathogen to the mucosa of the gastrointestinal tract by different mechanisms *(Candela et al., 2008; Ditu et al., 2011; Jin et al., 2000; Ouwehand and Conway, 1996)*. Jin et al. (2000) showed in an *in vitro* study with *E. faecium* 18C23 that the inhibiting effect on F4ac-positive *E. coli* (*E. coli* F4ac and F4MB) was not solely a pH effect since considerable inhibitory action was demonstrated after neutralizing to pH 7.0. The inhibition of adhesion of F4ac-positive *E. coli* by *E. faecium* 18C23 or its supernatant might occur through steric hindrance *(Jin et al., 2000)*. In another study Ditu et al. (2011) demonstrated that the susceptibility of seven enteropathogenic *E. coli* strains to aminoglycosides, beta-lactams, and quinolones was increased by *E. faecium* CMGB16 and decreased for amoxicillin-clavulanic acid. This investigation showed that the anti-infective effect of probiotics is also due to the modulation of virulence factors and antibiotic susceptibility expression in *E. coli* pathogenic strains *(Ditu et al., 2011)*. A recent published study reported that an increased pH improves the effect of aminoglycosides against pathogens *(Lebeaux et al., 2014)*. This could be yet another explanation: the probiotic simply influences the pH. Reduced binding could also result from antimicrobial agents produced by *E. faecium*, which may act against *E. coli*. However, a recent study found that none of the eight known bacteriocin genes are

present in *E. faecium* NCIMB 10415, and the strain shows no antimicrobial activity against Gram-negative bacteria *(Foulquie Moreno et al., 2003; Pollmann et al., 2005).* A competition for binding of the intestinal mucus receptor is also possible and has been shown for *E. faecium* strain 18C23 and F4-positive enterotoxigenic *E. coli* (*E. coli* F4ac and F4MB) in a model of porcine small intestine mucus *(Jin et al., 2000).*

Since *E. faecium* showed effects on the immune system there might be a connection between the intestinal microbial communities and the host's immune system. Twardziok et al. (2014) reported a strong crosstalk between both parts. Several associations between the intestinal microbial communities with the immune system were reduced through the feeding of *E. faecium*. Especially remarkable was the finding of the reduced association between microbial communities in the ileum and the expression of immune related genes in ileal Peyer's patches. Both targets were affected by the probiotic in 34-day-old piglets. This observation appears to support the possible immuno-modulatory effects of *E. faecium* NCIMB 10415.

Two hypotheses were postulated. The first one assumes that feeding with *E. faecium* can modify the composition of the microbial community, such that it relates less to the host immune system. The other hypothesis supposed that the supplementation moderates the response of the immune system to variations in the microbial communities. This is underlined by the result that post-weaning, *E. faecium*-supplemented piglets, which are subsequently infected with *Salmonella*, have to overcome the residual anti-inflammatory response/down-regulation in order to respond to the infection, resulting in a delay in the immune response *(Siepert et al., 2014).* The importance of the interplay between microbiota and the host immune response was already reported with the impact of the disturbance of one of both parts *(Buffie et al., 2013; Yeoman et al., 2014; Yurist-Doutsch et al., 2014).* In addition, pathogens like *E. coli* and *S.* Typhimurium have different virulence pathways and, accordingly, unique interactions with the host and its microbiota commonalities *(Yurist-Doutsch et al., 2014).* Probiotics like *E. faecium* may also act in different ways on the invading pathogen. Although certain microbes induce a proinflammatory response and others a tolerogenic one, how the immune response differentiates between them is unknown *(Yurist-Doutsch et al., 2014).* The challenge experiments with *Salmonella* revealed that a non-specific reaction of the immune system was activated under *E. faecium* supplementation and that there was no stimulation of the

immune response against *S.* Typhimurium by *E. faecium (Kreuzer et al., 2012a).* A possible hypothesis is that *E. faecium* sends signals to the immune system which reacts nonspecifically against it and induces a tolerance of the intracellular pathogen.

Formulated hypotheses, such as a reduction of the replication and proliferation within host cells or a faster clearance suggested for the intracellular pathogen chlamydiae *(Pollmann et al., 2005),* could not be verified in the studies of the "DFG Forschergruppe 483" and were not a topic of the CRC.

In conclusion, the probiotic *E. faecium* strain NCIMB 10415 showed positive and negative effects on the microbiota and the immune response of the host in the studies of the CRC 852, depending on time of observation and from which perspective the results are considered (e.g. microbiome, animal health, and animal performance). *E. faecium* showed positive effects against two different viruses and extracellular *E. coli*. For the intracellular pathogen *S.* Typhimurium *E. faecium* clearly showed a negative influence in regard to the amount of *Salmonella* able to reproduce in the animals. An overall study revealed that several associations between the intestinal microbial communities with the immune system were reduced by feeding the probiotic. This fact could be due to changes of the microbiota composition, which leads to other signaling and a non-specific immune response to *E. faecium*. Thus, *E. faecium* may induce a tolerogenic immune response against *Salmonella* by sending specific signals when binding to the mucus layer. For extracellular pathogens this tolerance reaction would not have any consequences if *E. faecium* blocked them through sterical hindrance. However, the specific mechanism by which *E. faecium* elaborates its effect still remains unclear and needs to be further investigated, e.g. in cell culture experiments. Nevertheless, it could be difficult if the effects depend on the interplay between the whole bacterial community and the immune system.

4.1.2. Zinc

Heavy metals, especially zinc, are often used as feed supplements for the promotion of growth and health in farm animals like pigs *(Castillo et al., 2008; Hojberg et al., 2005).* There are also pharmaceuticals for human use that contain zinc, which can be obtained in every pharmacy. Zinc is one of the most important trace elements and it has been

suggested it has a positive effect on the immune system *(Chasapis et al., 2012).* Many investigations were implemented to detect the effects and mechanisms of zinc on the microbiota and the host's immune system. Several of them were performed in pigs *(Hojberg et al., 2005; Janczyk et al., 2013; Jensen-Waern et al., 1998; Liu et al., 2014b; Starke et al., 2014; Vahjen et al., 2011).* Studies analyzing the effect of zinc on *E. coli* in piglets particularly also exist, because of the important role of *E. coli* in the weaning phase *(Crane et al., 2011; Katouli et al., 1999; Lee et al., 2005; Roselli et al., 2003; Sargeant et al., 2010; Vahjen et al., 2011).* It was the aim of this study to contribute to the knowledge about the effects of zinc to *E. coli* in the microbiota of pigs.

4.1.2.1. Effects on *E. coli*

The fact that zinc increases the proportion of a multi-resistant subpopulation in *E. coli* was an unexpected result (Publication 1). This kind of effect caused by zinc *in vivo* has not been previously reported or proven. Only a few studies exist that hypothesized that heavy metals like zinc contribute to the spread of antibiotic resistances in bacteria *(Cavaco et al., 2011; Holzel et al., 2012; Lee et al., 2005; Nishino et al., 2007).* Holzel et al. (2012) observed that environmental isolates of *E. coli* more frequently harbored antibiotic resistance against beta-lactams in a milieu like pig manure with an above average concentrations of copper and zinc. An *in vitro* study, in which *E. coli* was exposed to zinc, performed by Lee et al. (2005), demonstrated that zinc can modulate bacterial antibiotic resistance. They observed an up-regulated *mdt* operon (encoding a multidrug resistance pump) and many other genes influenced by zinc cultivation. Sixty four genes were significantly up-regulated by zinc stress, including genes known to be involved in zinc tolerance, particularly *zntA, zraP*, and *hydG*. Fifty eight genes were significantly down-regulated by zinc stress, whereby several of these genes were involved in protection against acid stress *(Lee et al., 2005).* Cavaco et al. (2011) suggested that the use of zinc in feed might have contributed to the emergence of MRSA, because zinc resistance and the *czrC* gene are widespread among CC398 MRSA isolates. Only recently a case-control study has actually proven that the amount of MRSA is associated with zinc feeding *(Slifierz et al., 2014).* It has been known for decades that zinc resistance may be linked to antibiotic resistance by co-selection. However, as we proved that the multi-resistant strains were not associated with zinc resistance, this is obviously not the main reason that explains the higher number of multi-resistant strains.

Resistance to copper in bacteria, in particular enterococci, is also often associated with resistance to antimicrobial drugs like macrolides and glycopeptides *(Amachawadi et al., 2011)*. However, it seems more likely that a resistance-driven effect is stronger at high heavy metal exposure and less effective at basal exposure levels *(Yazdankhah et al., 2014)*. There is also lack of knowledge concerning whether zinc-resistant bacteria may acquire antibiotic resistance genes, or if antibiotic-resistant bacteria are more capable of becoming zinc-resistant than antibiotic-susceptible bacteria *(Yazdankhah et al., 2014)*. Our study gives evidence that acquiring antibiotic resistance genes depends rather on a higher plasmid uptake rate than on the zinc resistance of bacteria.

In addition to the higher proportion of multi-resistant *E. coli*, it was highly interesting that zinc supplementation also led to an increased diversity of *E. coli* in the intestine of pigs (Publication 1) in contrast to the *E. faecium* supplementation trial (Publication 2). This corroborates findings by Vahjen et al. (2011). They demonstrated a higher overall enterobacterial diversity and that the number of species belonging to the family Enterobacteriaceae also increased in relative abundance in the small intestine of pigs fed with high zinc concentrations. Starke et al. (2014) observed in the same animal trial as we performed that most severe effects of high dietary zinc were observed one week after weaning in the stomach and small intestine. Reductions were observed for Entero-bacteriaceae and the *Escherichia* group as well as for *Lactobacillus* spp., especially for three of five studied *Lactobacillus* species. The impact of high dietary zinc diminished for enterobacteria with increasing age, but was permanent for *Lactobacillus* species *(Starke et al., 2014)*. It has already been shown that zinc supplementation of pigs contributed to the stability of the intestinal microbiota and the diversity of coliforms. However, this effect was limited to the first two weeks after weaning *(Katouli et al., 1999)*. Starke et al. (2013), who also participated in investigations during the CRC 852, showed that zinc leads to bacterial growth depression in the stomach and jejunum of weaned piglets in the early phase after weaning. Bacterial adaptation to zinc occurred within 2 to 3 weeks in piglets fed with a diet containing a pharmacological zinc oxide dosage. However, bacterial populations in older animals fed with a diet containing low zinc oxide also seemed to adapt to the presence of zinc during *ex vivo* growth *(Starke et al., 2013b)*. If zinc would select for zinc tolerant *E. coli* – why would the diversity increase? We would expect some bottleneck effect that should, in contrast, lead to reduced diversity. By taking into account the rapid

bacterial adaptation to dietary zinc it seems that the administration of zinc oxide in feed for weaned piglets might only be beneficial during a short period after weaning *(Starke et al., 2013b).*

In contrast to study 1 (results from VAG – PCR, not published), some studies also demonstrated an effect of zinc on the virulence of *E. coli* and other pathogens *(Bratz et al., 2013; Crane et al., 2014; Crane et al., 2007; Janczyk et al., 2013; Roselli et al., 2003; Slade et al., 2011).* It was shown that although zinc caused little or no inhibition of enteropathogenic *E. coli* (EPEC) growth, it led to a decrease in the expression of EPEC protein virulence factors, such as bundle-forming pili, EPEC secreted protein A, and other EPEC secreted proteins. Additionally, zinc reduced EPEC adherence to cells in tissue culture and the abundance of RNAs encoded by the *bfp* gene, by a plasmid-encoded regulator gene, by the locus for enterocyte effacement (LEE)-encoded regulator gene, and by several of the *esp* genes *(Crane et al., 2007). In vivo*, zinc reduced EPEC-induced fluid secretion into rabbit ileal loops, decreased the adherence of EPEC to ileum, and reduced histopathological damage. Some of the beneficial effects of zinc on EPEC infection appear to be due to the action of zinc on EPEC bacteria as well as on the host *(Crane et al., 2007).* Zinc also showed beneficial inhibitory effects against Shiga-toxin producing *E. coli* (STEC) strains that were parallel to those observed in EPEC *(Crane et al., 2011).* In addition, zinc strongly inhibited Shiga-toxin (Stx) expression. Stx is responsible for the extraintestinal effects of STEC infection, such as hemolytic-uremic syndrome (HUS). Zinc might be capable of preventing severe STEC infection *(Crane et al., 2011).* An *in vitro* study with ETEC showed that zinc may protect intestinal cells from ETEC infection by inhibiting the adhesion and internalization of bacteria, preventing the increase of tight junction permeability, and modulating cytokine gene expression *(Roselli et al., 2003). In vivo* zinc oxide (ZnO) supplementation could reduce the level of inflammation caused by an ETEC challenge. The supplementation was associated with a decrease in expression of immune response genes concerned with inflammation, and possibly related to the stage of infection. Interestingly, evidence was also obtained that a reduced level of MUC4 (a proposed ETEC F4 receptor) was associated with zinc supplementation, suggesting a mechanism that might influence ETEC infection *(Sargeant et al., 2010).*

In the CRC 852 it could be demonstrated that another intestinal pathogen, *Campylobacter (C.) coli*, showed a high susceptibility to zinc. High dietary ZnO levels in weaned piglets

reduced *C. coli* excretion significantly. There is evidence for the induction of an oxidative stress response by ZnO supplementation in *C. coli* and the reduction of the *C. coli* load by zinc could potentially lead to a lower contamination risk of meat during slaughter *(Bratz et al., 2013).*

Overall the ability of zinc to protect against enteric bacterial pathogens may be the result of its combined effects on host tissues, its inhibition of virulence in some pathogens, and the growth of different bacteria *(Bednorz et al., 2013b; Bratz et al., 2013; Crane et al., 2014; Vahjen et al., 2011).*

Some studies exist which report an influence of zinc on the immune system of the host *(Chai et al., 2014; Janczyk et al., 2013; Liu et al., 2014a; Sargeant et al., 2011).* Sargeant et al. (2011) showed that expression of genes involved in the innate immune response was reduced when cells were simultaneously exposed to ZnO. It was suggested that ZnO treatment inhibits the induction of NF-κB in response to pathogens, possibly through up-regulated heat shock proteins. A similar response *in vivo* with consecutive down-regulation in the inflammatory response would reduce further pathogen invasion and maintain normal gut function and growth *(Sargeant et al., 2011).* A study performed in the CRC 852 demonstrated that the mRNA expression of a toll-like receptor (TLR4) and the pro-inflammatory cytokine IL-8 were down-regulated in the high zinc group, while piglets fed with medium zinc concentrations had the highest expression. Liu et al. (2014) concluded that the dietary zinc level affected the colonic morphology, mucin profiles, and immunological traits in piglets after weaning. Those changes could support local defense mechanisms and influence colonic physiology and contribute to the reduction of post-weaning diarrhea *(Liu et al., 2014a).* Chai et al. (2014) showed that high dietary zinc could provide enhanced protection in the intestinal tract and stimulate the systemic humoral immune response against transmissible gastroenteritis virus infection. Another study observed that all positive effects disappeared after two weeks, and those rather negative effects, such as higher shedding of salmonellae, lower T-cell frequencies, and worse performance occurred. Hence, some authors postulated that supplementation with ZnO at high levels in the pig industry should be limited to 2 to 3 weeks *(Janczyk et al., 2013).*

All animals involved in the zinc feeding trial of the CRC 852 remained clinically healthy and diarrhea occurred only very rarely, with no differences between the feeding groups

(Starke et al., 2014). The performance of the piglets was improved by pharmacological zinc levels only for a short-term *(Martin et al., 2013b)*. High zinc concentrations increased intracellular zinc, promoted increased zinc export from intestinal tissues into extracellular compartments, and decreased zinc uptake from the gut lumen. The adaptive process appears to be established within 24 h *(Martin et al., 2013a)*. Intestinal mRNA levels of zinc transporters changed with high zinc supply, but this did not prevent zinc accumulation in tissues, suggesting hampered homoeostatic regulation. This might cause impaired performance during longer supply *(Martin et al., 2013b)*. The jejunal transport properties were not sustainably influenced in the post-weaning phase *(Gefeller et al., 2014)*. Starke et al. (2014) reported that zinc reduced bacterial metabolite concentrations and increased molar acetate ratios. Nevertheless, these differences diminished in older animals. Lower lactate concentrations were observed in the high dietary zinc group throughout the feeding trial *(Starke et al., 2014)*. Lower lactate concentrations could lead to reduced propionate concentrations and might therefore have an impact on hind gut microbiota, which could be modified due to reduced input of metabolites and a different bacterial species composition. This could imply consequences for bacterial species that require lactate as sole energy source *(Nagaraja and Titgemeyer, 2007)*.

Overall the results from all the different groups involved in the zinc feeding trial of the CRC revealed that the application of zinc in high concentrations leads to different transient and lasting effects during the development of the intestinal microbiota in piglets. Besides the effect of zinc on antibiotic resistance of some enterobacterial species, the composition of microbiota, the virulence of pathogens, and the host's tissue and metabolic activity were influenced.

Another critical aspect of using zinc as feed supplement in animals is its accumulation in the environment. Environmental isolates of *E. coli* were shown to be more frequently antibiotic resistance against beta-lactams in a milieu like pig manure with an above average concentration of copper and zinc *(Holzel et al., 2012)*. By contrast, the detection of mercury is associated with low antimicrobial resistance prevalence in *E. coli*. Thus, mercury contaminations in the environment could disperse the selection for antimicrobial resistance. However, relying on heavy metals to combat anti-microbial resistance would be like using a thief to catch a thief *(Holzel et al., 2012)*. Another study showed the

importance of a reduction of heavy metals in the future to prevent the further pollution of the environment *(Shi et al., 2011)*.

In summary, feeding with zinc showed a variety of different effects. Some of them might be positive, such as the piglets' higher weight gain in the first week after weaning and the higher diversity in the microbiota. However, zinc also had some critical effects like the spread of multi-resistance in *E. coli* and possibly also in other enterobacterial bacterial species. Furthermore, most positive effects were only detectable temporarily and heavy metals like zinc are also associated with risks for the environment. Most studies suggested that the feeding period for zinc should be no longer than two weeks post-weaning *(Janczyk et al., 2013; Katouli et al., 1999; Starke et al., 2014)*. However, negative effects, especially the higher proportion of multi-resistant *E. coli*, speak against the usage of high zinc concentrations in animal farming.

Possible mechanism of zinc on *E. coli* and the microbiota

The mechanism by which zinc affects the microbiota and the host's immune system is obviously complex. Various hypotheses exist about how zinc influences the microbiota of mammals especially. The most interesting and worrying finding is the induction of a higher proportion of multi-resistant *E. coli* by zinc. This may be explained by the two hypotheses presented in the following two paragraphs. A higher proportion of multi-resistant *E. coli* could be caused by an enhanced resistance plasmid uptake or by co-selection via zinc tolerance (Publication 1).

Zinc affects plasmid uptake in *E. coli*

Diverse hypotheses were created to explain an enhanced uptake of plasmid, carrying inter alia resistance genes, by *E. coli*. Stecher et al. (2012) showed that pathogen-driven inflammatory responses in the gut could generate transient enterobacterial blooms in which conjugative transfer occurred at extraordinary rates. These blooms may support reassortment of plasmid-encoded genes between pathogens and commensals, promoting the spread of fitness-, virulence-, and antibiotic-resistance determinants. Under normal conditions, HGT was blocked by the commensal microbiota, inhibiting contact-dependent conjugation between Enterobacteriaceae *(Stecher et al., 2012)*. However, as other members

of the CRC582 could not find any signs of increased inflammation in the piglets' guts, there is no indication that this hypothesis is relevant for the data we generated.
The unpublished data from Publication 1 demonstrated the opposite (see chapter 3.1.), namely that the total number and concentration of *E. coli* were not increased by zinc feeding. Although coliform numbers were not significantly changed by zinc supplementation (speaking against an enterobacterial bloom), a higher proportion of multi-resistant *E. coli* was observed, probably caused by an enhanced plasmid uptake. Most antibiotic resistance genes were detected on plasmids and could be exchanged by conjugative transfer (Publication 1). Other studies have previously suggested an influence of zinc on bacterial conjugation *(Holzel et al., 2012; Ou, 1973; Ou et al., 1972)* and on the structure of bacterial cell membranes by up-regulation of the expression of genes involved in cell membrane structure and membrane transport *(Lee et al., 2005).* Further investigations are needed to elucidate the question whether zinc has a direct influence on the HGT.

Zinc resistance of bacteria – one factor for a higher proportion of multi-resistance?

Enteral bacteria in livestock, both commensal and pathogenic, have been shown to develop resistance to trace elements such as zinc and copper. Due to this fact, strains with specific and high zinc tolerances could have a colonization advantage depending on their environment. In contrast, a low zinc tolerance could lead to an earlier toxic effect from zinc and other heavy metals *(Nies, 1999)* which are essential as trace elements for growth and maintenance.

Based on this assumption we verified whether the indirect effect of co-selection by zinc can be responsible for the higher proportion of multi-resistant *E. coli*. Antibiotic resistance genes may be physically linked to heavy metal resistance genes, e.g. on plasmids *(Bass et al., 1999; Cavaco et al., 2011; Ghosh et al., 2000).* Some multi-resistant clones detected were resistant to high zinc concentrations. However not all of them showed zinc resistance (Publication 1). Thus, co-selection of zinc resistance alone could not be the explanation for the higher proportion of multi-resistance in the *E. coli* population.

In contrast to our observations in *E. coli*, a study performed by Cavaco et al. (2011) found that MRSA strains from pigs had a high incidence of zinc resistance. This was mostly

associated with the *czrC* gene. The corresponding MSSA were susceptible to zinc. Slifierz et al. (2014) showed recently that ZnO is associated with an increase in the prevalence and persistence of MRSA among pigs. Their conclusion was a co-selection for methicillin resistance by ZnO. The use of ZnO as a growth promoter and therapy for colibacillosis would promote the persistence of MRSA in swine production *(Slifierz et al., 2014).* The source of methicillin zinc-resistant staphylococci in animals is unknown. It is not clear whether the methicillin-resistant staphylococci in animals are of human origin and have become resistant to zinc after exposure to the feed supplement or whether the zinc-resistant staphylococci have become resistant to methicillin due to exposure to antibiotics *(Cavaco et al., 2011).* To clarify this question additional investigations are required. These should include epidemiological studies based on whole-genome sequence data as well as on feeding trials including defined MSSA strains that could actually prove the mechanisms by which these resistances are selected for.

Although according to the data available zinc resistance is an unlikely explanation for the rise of multi-resistant *E. coli,* it may explain the higher diversity of the *E. coli* population which we showed with PFGE and MLST. Some normally dominant clones may be suppressed by high zinc amounts because of the lack of zinc resistance. Other strains with higher tolerance to zinc could then prevail and reach the detection limit. For the detection limit a frequency of at least 20% of a strain in a sample is needed, so that a 90% chance of detection exists *(Schlager et al., 2002).* Also, virulence of some bacteria could be influenced by a low zinc tolerance *(Bratz et al., 2013).* Furthermore, the expression of specific virulence genes may be suppressed by the pathogen itself to maintain the vital systems *(Crane et al., 2011; Crane et al., 2007).* However, we could not detect such effects because of low VAG numbers.

It is also possible that both hypotheses are true and that different mechanisms lead to the various effects of zinc.

4.2. Choice of animal model and experimental setup – critical factors

Several factors might have influenced the results of the studies. The main factor is the animal trial which is explained in Publications 1 and 2. Both feeding trials were organized similarly.

4.2.1. Animal trial

A critical aspect of the animal trial was the animal housing. Good and effective management of the piglets led to healthy animals (Publication 2) *(Starke et al., 2014)*. They were kept in pairs after weaning to reduce cage effects. However, this is not a normal procedure in commercial animal farming, where the pigs are always kept in large groups due to the factors of cost and space.

By keeping the piglets in pairs during the trial, this form of animal housing leads to a lower exchange of their microbial community by feces and to a higher individuality of their microbiota, compared to commercial pig farming. In order to prevent this, piglets from different sows were put together to form pairs. However, the results from counting *E. coli* showed a high individuality, leading to high dispersion of the values (see chapter 3.1). The animals harbored only a low number of strains with virulence associated genes, especially intestinal VAGs in general (Publication 2). This leads to difficulties in detecting any changes in the different feeding groups.

Another critical factor in the zinc feeding trial was that the sampling started one week after weaning, while zinc feeding started at weaning. Due to this fact, no data are accessible about the status of the pigs' microbiota, especially of *E. coli*, at the beginning of the zinc supplementation, or even before weaning. Therefore future studies should also investigate the *E. coli* population before weaning.

4.2.2. Sampling

Because of the comprehensive study design, excess workload, and limited capacity the sample sizes had to be reduced due to the excess workload and limited capacity. In the *E. faecium* trial samples were taken from *Colon ascendens* (mucosa and digesta) and feces. A statistical analysis of the three samples (mucosa, digesta, and feces) from the *E. faecium* trial showed no significant differences in their diversity (not published). Thus, the diversity

would not be affected, but single *E. coli* clones could not be detected. Therefore, the samples were reduced to digesta from Ileum and *Colon ascendens* of the three feeding groups (control, low, and high zinc group) from the zinc trial. After PFGE analysis the sample size was reduced to clones from the control and high zinc group.

4.2.3. Experimental setup

PFGE is time consuming and the analysis of the fingerprints with BioNumerics software, version 6.6 (Applied Maths, Belgium), is a standardized method. This method is highly discriminative and the risk of misinterpretation was reduced to a minimum by using only one person to do this analysis, thus limiting the influence of the human factor. PCR for detecting virulence-associated and antibiotic resistance genes is a normal and established procedure; nevertheless, it is prone to error. Today, it would be more effective to perform whole-genome sequence analysis (WGS) for the characterization of each clone. This could replace all PCRs detecting virulence or resistance genes and the MLST, and gives additional information about the strain.

4.3. Future Research

Future research is required for both nutritional supplements in order to gain more knowledge about the specific mechanisms and to verify or falsify our hypotheses and assure their safe and meaningful use in animal farming in the future. All samples and isolated *E. coli* clones from both animal trials were stored for future studies. Further investigations could be performed to verify the mechanisms of *E. faecium* on *E. coli* by *in vitro* studies in cell culture. Additional experiments with zinc could be performed to study zinc tolerance and the mechanism by which zinc enhances the plasmid exchange.

4.3.1. Probiotic *E. faecium*

For better characterization, all 168 clones of the control and probiotic group (Publication 2) could be analyzed by whole-genome sequencing. Adhesion assays using cell culture models could also show whether the clones from the probiotic supplemented group are less adhesive. Due to the good health status of the pigs during the trial, an additional trial implementing an infection model with *E. coli* could display clearer effects in the probiotic group, for example an aEPEC/EPEC or ETEC strain. Both pathotypes are important for the PWD in piglets and challenge studies already exist for ETEC. Although *E. faecium* showed positive effects, it is questionable whether *E. faecium* is suitable as a substitute for antibiotics, because of its lacking an effect on growth performance and feed intake *(Martin et al., 2012)*. Another model to enhance the positive effects of *E. faecium* could be the combination of different probiotic strains. A recent study showed that *E. faecium* combined with *Saccharomyces cerevisiae* have a better impact on pH regulation and maintenance of protozoa populations during subacute ruminal acidosis than *E.* faecium alone *(Chiquette et al., 2015)*. To go even further, the fecal microbiota transplantation (FMT) is another emerging therapy that regulates inflammation in experimental models. It was first used in the treatment of *C. difficile*-induced diarrhea *(Kassam et al., 2013)*. The principal of FMT for this indication is predicated on the concept that antibiotic therapy disrupts the normal ecology, allowing colonization of *C. difficile*. At present no controlled trials have yet been published *(Smits et al., 2013; West et al., 2015)*.

4.3.2. Zinc

Further investigation should also be performed on the specific mechanisms by which zinc induced a higher proportion of multi-resistant *E. coli in vivo*.

Whole-genome sequencing of the clones, especially of the subclones, could give more information about the strains. *In vitro* and *in vivo* studies on the bacterial conjugation under zinc conditions could contribute to the confirmation of the hypothesis that zinc affects the conjugation in *E. coli* and leads to enhanced plasmid uptake. This could be done by *in vitro* transformation studies with $ZnCl_2$ in culture media using a protocol of a transformation with $CaCl_2$. *In vivo* experiments in a mouse model with a donor and acceptor strain could further help to identify a mechanism of zinc on plasmid exchange. In addition, further studies on zinc resistance of the *E. coli* clones would be interesting and could give more information about whether zinc contributes to a higher diversity through a comparison of the clones from the control with those from the high zinc group. Zinc tolerance of each *E. coli* clone and subclone could be determined by a new established minimal inhibition concentration test with a minimal medium containing organic phosphate suitable for zinc experiments (e.g. $ZnCl_2$, which is better soluble in a water based medium).

Finally, other feed supplements could be tested for animal breeding to identify a better substitute for the prohibited antibiotics, because it seems that none of the tested supplements can adequately replace the antibiotics.

4.4. Conclusion

The aim of the project was to investigate the effects of the feed supplements *E. faecium* and zinc on the enteral *E. coli* microbiota in pigs. Currently, these supplements are used in animal breeding as replacement for antibiotic growth promotors, which were prohibited in the EU in 2006. We showed in two comprehensive studies characterizing more than 2,900 *E. coli* isolates in both feeding trials that both feed supplements affected the intestinal *E. coli* microbiota of pigs, but in different ways.

The probiotic *E. faecium* strain NCIMB 10415 influenced the *E. coli* population by reducing ExPEC-typical VAGs at the intestinal mucosa, while the diversity of *E. coli* remained unchanged. The specific influence of *E. faecium* on the mucosa showed that investigations of feces may be ineffective, as they do not provide a complete overview of the intestinal microbiota. Thus, our data question the relevance of fecal sampling only for studies on microbiota. In contrast to *E. faecium*, the divalent metal ion zinc caused a higher diversity of *E. coli* and an unexpected higher proportion of multi-resistant *E. coli*. The alarming result that zinc might promote the spread of antibiotic resistance calls into question whether zinc is an alternative to antimicrobials in animal farming.

In conclusion, neither of the investigated feed supplements seems to be optimal as feed supplement or a good candidate for the replacement of antibiotics as growth promoters in animal breeding. Although our results suggest a prophylactic effect of *E. faecium* against potential pathogenic *E. coli* at the mucosa, the lack of better performance of the piglets and the negative effect of a delayed immune response in *Salmonella* challenges do not provide motivation for the application of *E. faecium* as feed supplement for weaning piglets.

The data from the zinc trial showed that high zinc concentrations increase the proportion of multi-resistant *E. coli* dramatically. This must be considered as an unacceptable side effect, the worst thing that could happen, so to speak. The one and only argument for replacement of antibiotics as growth promoters with feed supplements like zinc should be the prevention of the spread of multi-resistances. In addition, zinc concentrations caused both short-term and lasting effects on the microbiota and the host. The environmental aspect should also be taken into account as zinc could more frequently induce antibiotic resistances in *E. coli*. Most studies recommended limiting the feeding of high zinc

concentrations to two weeks after weaning (post-weaning). However, if the evidence regarding a higher plasmid uptake promoted by zinc could be proven, feeding with high zinc dosages should be stopped, especially because multi-resistant clones were detected after one week of zinc supplementation.

5. Summary

Since the European Union prohibited antimicrobial growth promoters in livestock breeding in 2006, feed supplements like probiotics and bivalent cations have received more and more attention in animal farming. Pigs are the most relevant livestock population and supply the main part of meat consumed in Germany, which is a main global provider of slaughtered pigs. Furthermore, pigs are omnivores like humans and their gastrointestinal tract is similar to that of humans. Studying the intestinal microbiome in pigs is thus of particular importance not only for the pig population itself but also for understanding mechanisms of the human gut microbiome.

As a part of the CRC 852 the aim of this project (A3) was to investigate the impact of the feed supplements *E. faecium* NCIMB 10415 (a probiotic strain) and the divalent metal ion zinc on the intestinal *E. coli* population in pigs. *E. coli* is a member of the gastrointestinal microbiota of birds and mammals, including pigs, and contributes to the maintenance of the microbial gut balance. However, *E. coli* is also one of the most important intestinal pathogens in pig production, causing high economic losses, and is usually the cause of post-weaning diarrhea.

In two independent animal trials piglets were supplemented with the probiotic *E. faecium* NCIMB 10415 (*E. faecium* trial) and zinc oxide (zinc trial). Samples were taken from the intestine and the feces at different times around weaning.

In the *E. faecium* trial *E. coli* strains were isolated from three intestinal habitats (mucosa, digesta, and feces) of the probiotic and control group. *E. coli* bacteria were characterized via PFGE for clonal analysis. The high diversity of *E. coli* was reflected by 168 clones. MLST was used to determine the phylogenetic background. Pathotypes of *E. coli* were further defined using VAG-PCR. While these analyses discerned only a few significant differences in the *E. coli* population between the two feeding groups (*hlyF* [$P = 0.011$], *focG* [$P = 0.015$], *papC* [$P = 0.008$], *papGIII* [$P = 0.028$], *iroN* [$P = 0.04$], and *cvaC* [$P = 0.002$], less frequent in the probiotic group), analyses distinguishing clones that were uniquely isolated in the probiotic group only, the control group only, or both groups (shared group) revealed clear effects. Interestingly, extra-intestinal pathogenic *E. coli* (ExPEC)-typical clones adhering to the mucosa were significantly reduced in the probiotic

group (a total of 11 VAGs were reduced: *tsh* [*P* = 0.017], *mat* [*P* = 0.001], *focG* [*P* = 0.002], *papC* [*P* = 0.037], *colV* [*P* = 0.048], *ompT* [*P* = 0.003], *cvaC* [*P* = 0.004], *iroN* [*P* = 0.000], *etsB* [*P* = 0.003], *etsC* [*P* = 0.003], and *hlyF* [*P* = 0.001]). In addition, our data question the relevance of data based on only on the sampling of fecal *E. coli*. Using fecal samples only as a means of referring to the whole intestinal *E. coli* microbiota is questionable, because we could not detect any differences between fecal samples.

In the zinc trial, *E. coli* were isolated from ileum and colon digesta of the high zinc (2,500 ppm), low zinc, and control group (50 ppm). After counting, the *E. coli* population was characterized via PFGE and MLST for the determination of the phylogenetic background. Phenotypic resistance screening via ADT and MIC testing was followed with detection of resistance genes for selected clones. While the *E. coli* number did not change significantly (P = 0.787), a higher diversity of *E. coli* clones in animals supplemented with high zinc compared to the control group could be detected (36 clones in control group only vs. 69 clones in high zinc group only; 76 clones shared by both groups) after dividing the clones from high zinc and control group into three groups: clones appearing only in the control group (control only), clones appearing only in the zinc group (zinc only), and clones appearing in both groups (shared). The proportion of multi-resistant *E. coli* was significantly increased in the zinc group only compared to the control group only (18.6% vs. 0%). The shared group harbored 13.2% multi-resistant *E. coli*. Additionally, up to three additional phenotypic and genotypic resistances could be detected in subclones isolated from the high zinc group compared to their clones. Characterization of these subclones implied a higher proportion of antimicrobial resistance due to zinc's influences on plasmid uptake.

In conclusion, it seems that neither of the investigated feed supplements are optimal or suitable for the replacement of antibiotics as growth promoters in animal breeding. Although the results of this study imply a prophylactic effect of *E. faecium* against potential pathogenic *E. coli* at the mucosa, the lack of a better performance of the piglets and the negative effect of a delayed immune response in *Salmonella* challenges *(Kreuzer et al., 2012a; Siepert et al., 2014)* are an argument against an application of *E. faecium*. The results of the zinc trial showed that high zinc concentrations dramatically increase the proportion of multi-resistant *E. coli*. This is an unacceptable side effect, because feed supplements like zinc, used as replacement for antibiotics as growth promoter, should

prevent the spread of multi-resistances. If the hypothesis regarding a higher plasmid uptake promoted by zinc could be proven, feeding with high zinc concentrations should be stopped immediately.

6. Zusammenfassung

Seit dem die Europäische Union den Einsatz von antibiotischen Wachstumsförderern in der landwirtschaftlichen Tierproduktion im Jahre 2006 verboten hat, ist die Verwendung von Futterzusätzen kontinuierlich angestiegen. Dabei stellt die Schweinehaltung einen Hauptanteil in der deutschen Tierproduktion dar. Neben der wichtigen Rolle in der Fleischproduktion sind Schweine und deren Darmmikrobiota auch gut geeignet um Rückschlüsse auf die menschliche intestinale Mikrobiota zu ziehen. Bei beiden Spezies handelt es sich um Omnivore deren Anatomie des Verdauungstrakts sich sehr ähnelt. Aufgrund der Wichtigkeit in der Tierproduktion und der Ähnlichkeit zur menschlichen Darmmikrobita eignen sich Schweine sehr gut um Einflüsse von verschiedenen Futterzusätzen auf die intestinale Mikrobiota und das Immunsystem, das eng mit der Mikrobiota verbunden ist zu untersuchen.

Das Projekt war ein Teilprojekt (A3) des SFB 852 und hatte das Ziel den Einfluss der Futterzusätze *Enterococcus (E.) faecium* NCIMB 10415 (Probiotikum) und Zink auf die *E. coli* Mikrobiota in Schweinen zu untersuchen. Kommensale *E. coli* sind Bestandteil der Mikrobiota von Vögeln und Säugetieren und tragen zur Aufrechterhaltung der Darmbalance bei. Intestinal pathogene *E. coli* gehören zu den wichtigsten Pathogenen in der Mikrobiota und sind oft die Ursache von Durchfallerkrankungen bei Absetzferkeln. Die Fütterungsversuche sollten zeigen inwiefern die *E. coli* Population im Darm von den beiden Futterzusätzen beeinflusst wird.

In zwei unabhängigen Tierversuchen mit Ferkeln wurden jeweils das Probiotikum *E. faecium* NCIMB 10415 (*E. faecium* Versuch) und das zweiwertige Metallkation Zink in Form von Zinkoxid (Zink Versuch) gefüttert. Zu unterschiedlichen Zeitpunkten wurden unterschiedliche Proben aus dem Darm entnommen und der Kot der Ferkel gesammelt.

Im *E. faecium* Versuch wurden Proben vor und nach dem Absetzen entnommen (Mucosa und Digesta aus dem Colon und Kot), da das ein besonderer Stressauslöser bei den Tieren ist. Nach der Isolierung von *E. coli* aus den Proben und der Identifizierung von 168 Klonen wurden diese auf Unterschiede in ihrer Virulenz und Phylogenie untersucht. Dabei zeigten sich jedoch kaum signifikante Unterschiede zwischen den zwei Fütterungsgruppen (*hlyF* [$P = 0{,}011$], *focG* [$P = 0{,}015$], *papC* [$P = 0{,}008$], *papGIII* [$P = 0{,}028$], *iroN* [$P = 0{,}04$] und

cvaC [*P* = 0,002] waren in der Probiotikagruppe reduziert). Nach einer statistischen Analyse bei der die Klone nach deren Auftreten in drei Gruppen (Klone die ausschließlich in der Kontrollgruppe, der Probiotikagruppe und in beiden Fütterungsgruppen vorkamen) geteilt wurden, konnte nachgewiesen werden, dass die Fütterung von *E. faecium* die ExPEC-typischen Gene an der Mucosa reduzieren konnte (insgesamt 11 VAGs waren reduziert: *tsh* [*P* = 0,017], *mat* [*P* = 0,001], *focG* [*P* = 0,002], *papC* [*P* = 0.037], *colV* [*P* = 0,048], *ompT* [*P* = 0,003], *cvaC* [*P* = 0,004], *iroN* [*P* = 0,000], *etsB* [*P* = 0,003], *etsC* [*P* = 0,003] und *hlyF* [*P* = 0,001]). Zudem konnte gezeigt werden wie wichtig es ist Proben direkt aus dem Darm zu entnehmen, da in den Kotproben keine Unterschiede nachgewiesen werden konnten. Somit können Kotproben nicht die genauen Verhältnisse in der *E. coli* Mikrobiota wiedergeben. Damit müssen Studien zur *E. coli* Mikrobiota, die nur auf Kotproben basieren, in Frage gestellt werden.

Im Zinkversuch wurden die Proben (Digesta aus Ileum und Colon) erst nach dem Absetzen der Ferkel entnommen. Nach Auszählung und Isolierung der *E. coli* aus den Proben wurden 181 *E. coli* Klone mittels PFGE identifiziert. Nachdem genotypische Untersuchungen zum Nachweis von Virulenz-assoziierten Faktoren (VAG-PCR) und die MLST keine signifikanten Unterschiede im Auftreten von Virulenz und Phylogenie zeigen konnten (für alle getesteten Gene P-Werte $\geq$ 0,05), wurden die Klone mittels ADT und MIC auf Antibiotikaresistenzen untersucht. Während sich die *E. coli* Anzahl zwischen der hohen Zink- und Kontrollgruppe nicht signifikant unterschied (P = 0,787), konnte eine höhere Diversität von *E. coli* Klonen in den Tieren mit der hohen Zinkdosierung detektiert werden (36 Klone in der Kontroll- vs. 69 Klone in der hohen Zinkgruppe; 76 Klone, die in beiden Gruppen vorkamen), nachdem die Klone in drei Gruppen aufgeteilt wurden (Klone die nur in der Kontrollgruppe, nur in der Zinkgruppe und in beiden Gruppen vorkamen). Weiterhin zeigte der Vergleich der drei Gruppen, dass die Anzahl multiresistenter Klone in der Zinkgruppe erhöht war im Vergleich zur Kontrollgrupe (18,6% vs. 0%). Die Gruppe, die aus Klonen aus beiden Gruppen bestand, besaß 13,2% multiresistente *E. coli*. Zudem konnte nachgewiesen werden, dass Klone und deren Subklone, die in beiden Gruppen vorkamen, in der PFGE ein gleiches Profil aufwiesen, sich jedoch in der Anzahl an Resistenzen unterschieden. Mit einer Plasmidprofilanalyse und Southern Blotting konnte gezeigt werden, dass die meisten der Antibiotikaresistenzen auf Plasmiden codiert waren. Somit war die Fütterung von Zink mit einem erhöhten Anteil an multiresistenten *E. coli* im

Darm der Ferkel assoziiert. Ein Einfluss von Zink auf den Plasmidaustausch, der diesen erhöhen würde, könnte dabei eine mögliche Ursache sein.

Im Rahmen dieser Arbeit wird deutlich, dass keines der beiden Futterzusätze als optimaler Ersatz für Antibiotika als Wachstumsförderer in der Tierproduktion geeignet ist. Obwohl unsere Ergebnisse auf einen prophylaktischen Effekt von *E. faecium* gegen potentiell pathogene *E. coli* an der Mukosa hinweisen, sprechen die fehlende Leistungssteigerung der Ferkel (Futteraufnahme, Wachstum) und der negative Einfluss auf die Immunantwort während der Salmonellen Infektionsversuche *(Kreuzer et al., 2012a; Siepert et al., 2014)* gegen eine Applikation von *E. faecium*. Der Zinkversuch hat gezeigt, dass hohe Zinkkonzentrationen mit einem dramatisch erhöhten Anteil an multiresistenten *E. coli* einhergehen. Dies ist ein unakzeptabler Nebeneffekt, da der wesentliche Grund für das Verbot von Antibiotika als Wachstumsförderer und die daraus resultierende Nutzung von Futterzusätzen wie Zink, die Verhinderung der Anreicherung und Verbreitung von Multiresistenzen war. Falls sich die Annahme bestätigt, dass Zink eine erhöhte Plasmidaufnahme fördert, sollte die Fütterung von hohen Zinkdosen sofort gestoppt werden.

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Zhou, Z., McCann, A., Litrup, E., Murphy, R., Cormican, M., Fanning, S., Brown, D., Guttman, D. S., Brisse, S., and Achtman, M. (2013): Neutral genomic microevolution of a recently emerged pathogen, *Salmonella enterica* serovar Agona. PLoS Genet 9, e1003471.

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8. List of Publications

8.1. Publications

8.1.1. Peer-reviewed

Bednorz C, Oelgeschläger K, Kinnemann B, Hartmann S, Neumann K, Pieper R, Bethe A, Semmler T, Tedin K, Schierack P, Wieler LH, Guenther S. (2013):
The broader context of antibiotic resistance: zinc feed supplementation of piglets increases the proportion of multi-resistant *Escherichia coli* in vivo.
Int J Med Microbiol. 2013 Aug; 303(6-7): 396-403.

Bednorz C, Guenther S, Oelgeschläger K, Kinnemann B, Pieper R, Hartmann S, Tedin K, Semmler T, Neumann K, Schierack P, Bethe A, Wieler LH. (2013):
Feeding the probiotic *Enterococcus faecium* strain NCIMB 10415 to piglets specifically reduces the number of *Escherichia coli* pathotypes that adhere to the gut mucosa.
Appl Environ Microbiol. 2013 Dec; 79(24): 7896-904.

S. O. Twardziok, R. Pieper, J. R. Aschenbach, C. **Bednorz**, G. A. Brockmann, M. Fromm, S. Klingspor, S. Kreuzer, U. Lodemann, H. Martens, L. Martin, J. F. Richter, L. Scharek-Tedin, B. F. Siepert, I. C. Starke, K. Tedin, W. Vahjen, L. H. Wieler, S. S. Zakrzewski, J. Zentek and P. Wrede (2013):
Cross-talk Between Host, Microbiome and Probiotics: A Systems Biology Approach for Analyzing the Effects of Probiotic *Enterococcus faecium* NCIMB 10415 in Piglets.
Molecular Informatics Volume 33, Issue 3, pages 171–182, March 2014.

8.1.2. Non peer-reviewed

Carmen Bednorz, Sebastian Guenther und Lothar H. Wieler (2014): Einsatz von Probiotika und Zink als Futterzusätze in der landwirtschaftlichen Schweinehaltung. Tierärztl. Umschau 69, 000 – 000 (2014).

8.2. Oral presentations

Bednorz, C, Guenther, S, Wieler, LH (2013). Zinc feed supplementation of piglets increases the proportion of multi-resistant *Escherichia coli in vivo*. Minisymposium SFB 852, Berlin, Germany (12.04.2013).

Bednorz, C, Guenther, S, Wieler, LH (2011). Effects of zinc and probiotics on the dynamics and function of *E. coli* populations in the swine gut, DRS-Doktorandensymposium 2011, Berlin, Germany (30.06.2011).

Bednorz, C, Guenther, S, Wieler, LH (2011). Effects of zinc and probiotics on the dynamics and function of *E. coli* populations in the swine gut, Minisymposium Biology of Nutrition, SFB852 Berlin, Germany (02.05.2011).

Bednorz, C, Guenther, S, Wieler, LH (2010). Description of effects of feed additives and probiotics on the dynamics of *E. coli* populations in the swine gut and their functional characterization *in vitro*, Summer School SFB 852, Berlin, Germany (18.08.2010).

Bednorz, C, Guenther, S, Wieler, LH (2010). Effects of zinc and probiotics on the dynamics and function of *E. coli* populations in the swine gut, Minisymposium SFB 852, Berlin, Germany (11.06.2010).

8.3. Poster presentation

Bednorz, C, Oelgeschläger, K, Kinnemann, B, Hartmann, S, Neumann, K, Pieper, R, Bethe, B, Schierack, P, Tedin, K, Wieler, LH, Guenther, S (2013): Increased diversity and antimicrobial resistance of *Escherichia coli* populations in the gut of piglets under zinc supplementation, 1st International Workshop on Nutrition and Intestinal Microbiota – Host Interaction in the Pig, FU Berlin, Berlin, Germany, ISBN: 978 3 00 043962 9, (24.-25.10.2013).

Bednorz, C, Oelgeschläger, K, Kinnemann, B, Neumann, K, Pieper, R, Bethe, A, Schierack, P, Tedin, K, Guenther, S and Wieler, LH (2013). Zinc feed supplementation increases diversity and antimicrobial resistance of *Escherichia coli* populations in the gut of piglets, 5th Congress of European Microbiologists, FEMS, Leipzig, Germany (21.07.– 25.07.2013).

Bednorz, C, Guenther, S, Oelgeschläger, K, Kinnemann, B, Pieper, R, Hartmann, S, Tedin, K, Semmler, T, Neumann, K, Schierack, P, Bethe, A, Wieler, LH (2013): Effect of the probiotic *Enterococcus faecium* strain NCIMB 10415 on *Escherichia*

coli populations in the gut of weaned piglets, 5th Congress of European Microbiologists, FEMS, Leipzig, Germany (21.07.-25.07.2013).

Carmen Bednorz; Bianca Kinnemann, Kathrin Oelgeschläger, Sebastian Guenther; Lothar H. Wieler (2012). Effects of zinc and the probiotic *E. faecium* on the dynamic and function of *E. coli* populations in the swine gut, Minisymposium SFB 852, Berlin, Germany (12.04.2012).

Carmen Bednorz; Kathrin Oelgeschläger; Bianca Kinnemann; Robert Pieper; Sebastian Guenther; Lothar H. Wieler (2012). Effect of *E. faecium* on the diversity of *E. coli* populations in the swine gut, DVG-Fachtagung Bakteriologie und Mykologie, Leipzig, Germany (27.-29.06.2012).

Curriculum Vitae

For reasons of data protection,

the curriculum vitae is not included in the online version

Selbstständigkeitserkärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit ohne die unzulässige Hilfe Dritter und ohne die Verwendung anderer als der angegebenen Hilfsmittel angefertigt habe.
Diese Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Berlin, 26. Februar 2015